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EXPERIMENTAL RAT MODELS TO STUDY ALCOHOLIC LIVER DISEASE

Nutrition-dependent alterations of liver biochemistry and morphology

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Stellingen

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EXPERIMENTAL RAT MODELS TO STUDY ALCOHOLIC LIVER DISEASE:
NUTRITION-DEPENDENT ALTERATIONS
OF LIVER BIOCHEMISTRY AND MORPHOLOGY

1

De mate van toxiciteit van alcohol voor de lever wordt mede bepaald door het dieet.

Dit proefschrift

2

Het gehalte aan vitamine A in de lever heeft een belangrijke invloed op de ontwikkeling van liverfibrose.

Dit proefschrift

3

Er kan niet zonder meer gesteld worden dat ook een matig alcoholgebruik slecht is voor de lever.

Dit proefschrift

4

Het belang van vitamines voor de instandhouding van een goede gezondheid is onderkend. Het is echter enigszins overdreven om een pasgeborene vitamine K toe te dienen nog voordat de ouders het kind hebben vastgehouden (Ziekenhuis "Rivierenland" te Tiel, 10 december 1992, 15.07 uur).

5

Proefdierkunde is de enige leerstoel die zichzelf probeert op te heffen.

6

Bij de berekening van de energiebesparing van een spaarlamp moet rekening worden gehouden met de verhoogde stookkosten die het gevolg zijn van de geringere warmteafgifte van de spaarlamp ten opzichte van de gloeilamp.

7

Men dient zich te realiseren dat het gevoel van geluk gerelateerd is aan het gevoel van ongeluk en dat beide nodig zijn voor een individu om te overleven.

8

Voor de opzet en uitvoer van wetenschappelijk onderzoek is creativiteit gewenst, voor het regelen van kinderopvang in Nederland is creativiteit noodzakelijk.

9

Huidige werkgevers die illegalen in dienst hebben zijn te vergelijken met de Amerikaanse boeren van voor de Burgeroorlog die slaven bezaten voor hun persoonlijke gewin.

10

Een Verenigde Staten van Europa is een illusie zolang zelfs de burgers van de natie Duitsland zich onderling blijven onderscheiden als "Ossies" en "Wessies".

Leiden, 25 februari 1993
Willem Friedrich Seifert

EXPERIMENTAL RAT MODELS TO STUDY ALCOHOLIC LIVER DISEASE

Nutrition-dependent alterations of liver biochemistry and morphology

Proefschrift

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aan de Rijksuniversiteit te Leiden,
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hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
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Willem Friedrich Seifert

geboren te Schiedam in 1960

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Aan mijn ouders

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Alcohol (= ethanol) is both a drug and a nutrient. Alcohol is consumed for its mood-altering effects and is thereby a psycho-active drug. Alcohol is rich in energy and in many societies alcoholic beverages are a normal component of the basic food supply. A large intake of alcohol, for whatever reason, results in various toxic effects. Alcohol abuse can adversely affect almost all organs of the body, amongst which the liver is particularly susceptible to injury because it is the primary site for ethanol oxidation.

Over the past decades there has been a steady increase in the per capita consumption of alcohol in the Western world. In the Netherlands, per capita consumption of absolute alcohol has been increased from 2.1 litres in 1950 to 8.3 litres in 1987 (1) In 1990 and 1991 a small decrease to 8.0 litres was noticed (2). Numerous studies from many parts of the world have demonstrated a clear correlation between the average quantity of alcohol consumed and mortality from cirrhosis of the liver (3,4,5). Population studies have shown that an increase in alcohol consumption in a population correlated with an increase in the frequency and mortality of alcohol-related diseases (3). The estimated number of alcohol-related deaths in the Netherlands increased by 150 percent between 1960 and 1985 (1). At the same time, deaths from liver cirrhosis increased by 32 percent.

Unless mankind refrains from drinking, which is unlikely in view of the lack of results from efforts to alter current drinking attitudes, specific intervention is necessary to combat alcoholic liver disease. Up till now, no therapy is available despite intensive research on the effects of alcohol on the liver. This is due to a failure in understanding the exact pathogenesis of alcoholic liver disease which partly relates to conflicting results from the many studies concerning the effects of alcohol on liver function.

This chapter presents a discussion of current knowledge concerning the hepatic metabolism of alcohol, the effects of alcohol on hepatic function and structure, the inter-relationships between alcoholism, (mal)nutrition and liver disease as well as the animal models which are used to study the pathogenesis of alcoholic liver disease.

1.2 Ethanol metabolism

Ethanol is quickly absorbed from the gastrointestinal tract into the blood. On its way through the body, approximately ten percent of the amount absorbed is metabolized by the stomach or eliminated through kidneys and lungs; the remaining part is oxidized in the body, mainly in the liver. Extrahepatic metabolism of ethanol is limited.

The liver contains three different enzymatic systems for ethanol oxidation, each located in a different subcellular compartment of the parenchymal cells: alcohol dehydrogenase (ADH) in the cytosol, microsomal ethanol oxidizing system (MEOS) located in the endoplasmic reticulum and catalase located in the peroxisomes. Acetaldehyde is the product of each of these oxidative reactions.

Alcohol Dehydrogenase Pathway (ADH)

The enzyme ADH catalyses the oxidation of ethanol to acetaldehyde (6). This conversion requires the cofactor nicotine-amide-adenine dinucleotide (NAD), which is the acceptor of the hydrogen (H). The net result of the ethanol oxidation is an increased NADH:NAD ratio in the cytosol of the parenchymal cell, primarily because the metabolic systems are not able to oxidize NADH.

Microsomal Ethanol Oxidizing System (MEOS)

A second pathway for ethanol oxidation in the liver is the cytochrome P-450 dependent microsomal ethanol oxidizing system (7,8). MEOS is localized in the smooth endoplasmic reticulum and utilizes cytochrome P-450, NADPH-cytochrome P-450 reductase and O_2 to oxidize ethanol to acetaldehyde. The quantitative role of this system is probably low under normal conditions since MEOS requires higher ethanol concentration for half-maximal activity (Michaelis constant) than does oxidation mediated by ADH (10-15 mM and 0.2-2 mM ethanol, respectively (9)). However, MEOS may be important at high blood ethanol levels or during chronic use of ethanol. It has been observed in rats (10) and in humans (11) that long-term ethanol consumption is associated with the proliferation of hepatic microsomal membrane and with an increase of hepatic MEOS activity. This implicates a role for MEOS in inducing metabolic tolerance to ethanol.

Ethanol may induce a specific cytochrome P-450 isoenzyme in rats (12) and in rabbits (13). There are also indications of the existence of the ethanol-inducible form of cytochrome P-450 in humans (14,15).

Catalase Pathway

The peroxisomal enzyme catalase oxidizes ethanol to acetaldehyde *in vitro* in the presence of an H_2O_2-generating system (16). However, the H_2O_2-mediated ethanol peroxidation is believed to play only a minor part *in vivo*, because its activity depends on

the availability of hydrogen peroxide which is low under normal circumstances in the liver (17).

Acetaldehyde is further converted to acetate by aldehyde dehydrogenase, which is located in liver mitochondria of rodents (18), baboons (19) and humans (20). NAD is also the coenzyme in this reaction step and it is oxidized to NADH. Under normal conditions, acetate is the final product of the oxidation of ethanol in the liver and it is released into the blood stream. Enzymes in peripheral tissues further oxidize acetate to CO_2 and H_2O.

1.3 Hepatoxicity of ethanol

Effects on electron transport and oxidative phosphorylation

Ethanol oxidation can be an useful energy source for the cell when it is coupled in some way to adenosine triphosphate (ATP) production. During ADH- and aldehyde dehydrogenase-catalysed oxidation of ethanol and acetaldehyde, respectively, NADH is formed as an energy rich intermediate that can be used for synthesis of ATP in mitochondria through the processes of electron transport and oxidative phosphorylation. However, there are indications that the hepatic ATP content is reduced during chronic ethanol feeding (21,22,23). This decrease could be due either to increased utilization of ATP in response to increases in hepatic ATP-ase activity or to decreased synthesis of ATP. Increased activity of hepatic ATP-ase would be expected to increase ATP utilization by the liver, in effect creating a hypermetabolic state. Israel and co-workers tested this hypothesis and observed consistent increases of oxygen consumption by the liver with an increase in ATP-ase activity following chronic ethanol administration (24).

Decreased ATP synthesis has been reported in animals fed ethanol for more than one month. Gordon reported that this decrease resulted from the accumulation of long-chain Co-enzyme A (CoA) derivatives of fatty acids in alcohol-induced fatty liver, which impede translocation of adenosine diphosphate (ADP) into mitochondria and thereby limit the rate of oxidative phosphorylation (25). Thayer and Rubin observed a 35-40% decrease in ATP synthesis in isolated submitochondrial particles from livers of rats fed ethanol for one month (26). These results indicate that the decrease in respiration are a consequence of alterations in the activities of the enzymes of the electron transport chain themselves. The activities of cytochrome oxidase, cytochrome b and NADH dehydrogenase per mg mitochondrial protein were decreased to 40-50% of those in control livers. Other studies have found decreases in respiration in intact mitochondria after chronic ethanol feeding in both rats and baboons (22,23,27,28). In these studies, ADP-stimulated (state-3) oxygen consumption was decreased, whereas basal (state-4) oxygen consumption was unchanged (23,28). The decreased ATP synthesis was related

to the decreased catalytic activity of the ATP synthethase complex as measured by the ATP-P_i exchange rate (22,28), rather than to alterations in the proton gradient. Mitochondria also showed morphological evidence of damage; they were present as megamitochondria and exhibited disorganization of cristae, even at the stage of fatty liver. Morphological changes of this type have also been observed in the parenchymal cells of alcoholics (29).

Effects of acetaldehyde

All three oxidative pathways of ethanol metabolism result in the formation of acetaldehyde. Acetaldehyde is very reactive and is able to form stable adducts with proteins. It has a high affinity for sulphhydryl groups which makes proteins which are rich in cysteine and lysine more vulnerable to acetaldehyde toxicity. It has been reported that it selectively forms a stable adduct in rat liver microsomes with the ethanol-inducible P-450 isoenzyme (30). Furthermore, covalent binding to other hepatic macromolecules (31), plasma proteins (32,33) and cytoskeletal proteins such as tubulin (34,35) has been observed.

The pathogenesis of the liver abnormalities may be the result of adverse effects of acetaldehyde. Two features of primary alcoholic hepatic damage are the deposition of fat and the enlargement of the liver. It has been shown that both lipids and proteins account for the increase in the dry weight of the liver (36). The increase of proteins is partly due to the acetaldehyde-induced impairment of the protein secretion mediated by microtubules. Microtubules are formed by polymerization of tubulin, a process in which the sulphhydryl groups of cysteine residues are involved. Long-term ethanol feeding results in a decrease both in the concentration of total tubulin (36) and in the polymerization of tubulin to microtubules (34,35). In these livers, the secretion of proteins such as transferrin and albumin was impaired. The rise in intracellular proteins results in larger parenchymal cells in association with a reduction in intercellular space, increased sinusoidal compression and portal hypertension.

Other toxic effects of acetaldehyde have been linked to lipid peroxidation (37). The binding of acetaldehyde with cysteine, or with cysteine-containing glutathione, or with both, may contribute to depression of liver glutathione (38), thereby reducing the scavenging of toxic-free radicals by this peptide. There are indications that specific glutathione reduction which occurs in alcoholics (39) can be prevented by the administration of methionine (38), a precursor of cysteine and glutathione. There is increasing evidence for the involvement of free radicals in the pathogenesis of alcoholic liver disease and for the role of free radicals in both initiation and progression of ethanol-induced liver damage (40,41). The role of free radicals in lipid peroxidation, with the consequent disturbances of membrane function and formation of hydroxyl and superoxide-free radicals has been demonstrated during both alcohol and aldehyde oxidation.

Effects of MEOS on the activation of xenobiotic agents and micronutrients

The cytochrome P-450 dependent MEOS plays a major role in multiple ethanol-drug interactions. In view of its inducibility and broad substrate specificity, it increases the susceptibility of alcoholics to the hepatotoxic effects of various xenobiotic agents. Acute and chronic effects of ethanol on drug metabolism have been described.

One acute effect of the presence of ethanol is the inhibition of hepatic drug metabolism (42,43,44). This may occur either by direct competition between ethanol and other drugs for the metabolic pathway involving cytochrome P-450 of MEOS (42,43) or by indirect competition between ethanol oxidation processes and drug detoxification processes for the supply of NADPH in the microsomes (44). For example, ethanol competes with acetaminophen for its microsomal metabolism and, in this way, it suppresses the biotransformation of acetaminophen to toxic metabolites (45).

Chronic ethanol consumption results in a metabolic tolerance for ethanol which is due to an increased activity of non-ADH pathways. This seems to be related to an increase in the activity of MEOS (see 1.2). In addition to tolerance to ethanol, tolerance to various other drugs has also been associated with the induction of MEOS activity by ethanol pre-treatment. The metabolic drug tolerance persists for a period of several days to weeks after the cessation of ethanol abuse. The duration varies with each drug (46). In both man and rat, ethanol consumption resulted in a striking increase in the rate of blood clearance of certain drugs, such as meprobamate and phenobarbital (47), tolbutamide (48) and propanolol (49).

The increase in microsomal enzyme activity due to chronic ethanol ingestion also applies to the conversion of exogenous compounds into toxic metabolites. Consequently, chronic alcohol intake has been shown to enhance vulnerability to hepatotoxic agents. For instance, carbon tetrachloride (= CCl_4,(50)) and acetaminophen (51) exert their toxicity in rats and rabbits after conversion to a reactive metabolite in the microsomes. Both drugs possess a high affinity for the ethanol-inducible form of cytochrome P-450 of MEOS and indeed, alcohol pre-treatment remarkably enhances their hepatotoxicity (52,53).

The induction of the microsomal oxidative pathway by ethanol alters the micronutrient metabolism of liver and other tissues. For instance, vitamin D, structurally related to ethanol, may serve as a substrate for the microsomal enzymes (54). It has also been shown that vitamin A can serve as a substrate for microsomal oxidases. A pathway specific for retinol and retinoic acid metabolism has been discovered in microsomes (55,56). Furthermore, a microsomal NAD^+-dependent retinol dehydrogenase has been described (57). This pathway can be induced by ethanol and by certain drugs and has been linked to the clinical occurrence of hepatic depletion of vitamin A in alcoholics (58, see 1.6.1). As a consequence, vitamin A metabolism plays an important role in ethanol-related diseases; for instance, ethanol-induced vitamin A deficiency of the liver and

plasma leads to night blindness and sexual dysfunction in alcoholics (59). Furthermore, it enhances potential liver dysfunction. The role of vitamin A during chronic ethanol and CCl_4 administration, particularly during liver fibrosis, has been further investigated in this thesis (Chapter 2 and 5).

1.4 Liver cells involved in alcoholic liver disease

1.4.1 Structure and cellular composition of the normal liver

The structural unit of the liver is the lobule. It is composed of the liver parenchyma surrounding one central, or efferent, hepatic vein and of several portal tracts at its periphery. The architecture of the lobular parenchyma shows a radial arrangement of single parenchymal cell plates lined by sinusoidal blood spaces (sinusoids) draining into the central hepatic vein. The acinus has the portal tract in its center and consists of parts of several lobules. Physiologic considerations and histochemical localization of various hepatic functions make the acinus a more reasonable functional unit than is the lobule. The blood supply of the lobule originates from the portal tract vessels: approximately 80% from the branches of the portal vein and 20% from the hepatic artery. In addition to blood vessels, portal tracts contain lymph vessels, nerves and bile ducts.

The liver lobule consists of several cell types outside the portal tracts. The large parenchymal cells, or hepatocytes, represent 60% of the total cell number in rat liver, occupy 80% of the liver volume and contain 93% of the liver protein content (60). The parenchymal cell is responsible for most of the main liver functions.

Non-parenchymal cells represent a total of 40% of the number of cells and occupies only 6.5% of the liver volume. The main non-parenchymal cell types are endothelial cells, fat-storing cells, Kupffer cells and pit cells. The sinusoids are lined by endothelial cells. Endothelial cells of the liver contain fenestrations and in this respect they differ morphologically from other endothelial cells (61). Fat-storing cells are localized underneath the sinusoidal lining. They play a role in vitamin A metabolism and storage, and in the synthesis of extracellular matrix components (62,63). Kupffer cells are situated in the sinusoids preferentially around branches of the portal veins. The functions of Kupffer cells include phagocytosis of particulate material, detoxification of endotoxin, secretion of mediators, regulation of the hepatic microcirculation, processing of antigens, mediation of various immune reactions, and uptake and catabolism of lipids and glycoproteins including many enzymes (64). Pit cells are located on, or embedded in, the endothelial cell lining. Since pit cells have a natural cytotoxic activity against tumours, they may play a role in the hepatic defence against metastatic tumours (65).

The remainder of the liver volume is formed by the extracellular spaces (60).

1.4.2 Parenchymal cells

The parenchymal cells are the largest of the liver cells. They are involved in a number of different important metabolic pathways. These include specific liver functions such as energy metabolism, urea synthesis, ammonia metabolism, lipoprotein metabolism, bile production, detoxification of xenobiotics and synthesis of plasma proteins. These numerous metabolic functions are supported by a specific architectural arrangement and a specific cell organelle composition. In human liver, there are approximately 2,000 mitochondria per parenchymal cell, and they account for 18% of the total cellular volume (66). Mitochondria are more numerous, larger and oblong in parenchymal cells around portal tracts, and are closely related to the rough endoplasmic reticulum, which comprises approximately 7% of cellular space. The smooth endoplasmic reticulum and the Golgi apparatus constitute 12% of cellular volume. The number and size of lysosomes vary greatly. There are about 1,000 peroxisomes in each parenchymal cell.

The parenchymal cells of an acinus have been divided into three distinct zones according to their proximity to the portal venules (67). The acinar zone I closely surrounds the terminal portal venule, zone III is located around the hepatic vein and zone II occupies an intermediate position between the two. A number of histochemical and biochemical measurements, either in situ or following microdissection, have shown functional, morphological and biochemical heterogenity between parenchymal cells of an acinus (67).

Heterogeneous distribution over the three zones of different enzymes involved in the metabolism of carbohydrates, proteins, lipids and xenobiotics has been reported (67). Gluconeogenesis predominates in the acinar zone I, whereas glycolysis mostly occurs in parenchymal cells of zone III. Also ureogenesis from ammonia is located mainly in zone I, but the synthesis of glutamine takes place preferentially in zone III. Ethanol and carbon tetrachloride detoxication occurs primarily in the parenchymal cells of the perivenular region or zone III of the hepatic acinus.

Alcoholic liver injury develops in the parenchymal cells of zone III. Two mechanisms have been proposed to explain the enhanced toxicity of ethanol in zone III. The first concept is that ethanol oxidation exacerbates the pre-existing relative hypoxia in the perivenular region by increasing oxygen consumption (68). The second proposed mechanism is that the lowered PO_2 in the perivenular parenchymal cells exaggerates ethanol toxicity by interfering with oxidation of the excessive reducing equivalents generated during ethanol oxidation (69). Hepatic anoxia and the redox shift caused by ethanol oxidation may explain the selective damage and necrosis of parenchymal cells in the perivenular zone.

The first signs of liver damage by ethanol is swelling of parenchymal cells. Swelling of the parenchymal cells has been attributed to fat deposition. Fatty liver (steatosis) and

its associated hepatomegaly have been considered a benign condition which is reversible with alcohol abstinence (70). The mechanism of hepatomegaly clearly involves more than just fat deposition. It has been shown that hepatomegaly is the result of increased parenchymal cell size and not of parenchymal cell number (36). Total fat accounts for only 20 to 30% of the increase in liver weight in hepatomegaly and increased total protein content has been reported to account for approximately another 20%. The other 50 to 60% increase in liver weight is due to the accumulation of water.

Parenchymal cell expansion, resulting in compression of the sinusoidal area, has been associated with portal hypertension (71). Portal hypertension can occur as a relatively early manifestation of alcoholic liver disease and parenchymal cell swelling appears to be an important determinant in its development. However, it should be stated that cirrhosis with nodule formation and fibrosis play a major role in causing or sustaining portal hypertension (70).

The injured parenchymal cells (see 1.3) may contain megamitochondria (27) and Mallory bodies (72). Megamitochondria contain enlarged and abnormal cristae, paracrystalline inclusions, and disrupted membranes. The functional significance of these alterations in mitochondria is controversial. Videla *et al.* (73) found no change in mitochondrial activity despite chronic ethanol exposure, while a study by Jenkins and Peters (74) demonstrated that hepatic mitochondria of alcoholics with mild forms of liver disease have normal or increased activity of a number of mitochondrial enzymes. Megamitochondria are, however, not specific to alcoholic liver disease and are occasionally found in normal parenchymal cells (75).

Mallory bodies are a characteristic morphological abnormality found in human alcoholic hepatitis (72). However, they are not specific for alcoholic liver disease and are regularly found in parenchymal cells of patients with non-alcohol related diseases e.g. Wilson's disease and chronic cholestatic syndromes. Mallory bodies consist of non-branched, 15 to 20 nm tubular, filamentous rods, covered by a fimbriated surface coat (76). Connections of Mallory bodies with the plasma membrane and vesicles have been reported. These structural connections may be important both in terms of the origin of Mallory bodies and of their contribution to cellular malfunction. There are three hypotheses regarding the pathogenesis of Mallory bodies (72). The first concept states that Mallory bodies result from vitamin A deficiency states which induce pathological keratinization within parenchymal cells (72). The second theory proposes a defect in microtubular assembly and the third, the preneoplasia hypothesis, suggests that "structural phenotypic cellular changes" such as those associated with neoplasm are responsible for Mallory body formation (72). Changes in cytoskeleton with formation of Mallory's alcoholic hyalin imply alteration of the tubulin system. This may cause retention of proteins, such as albumin and transferrin, producing hydrophobic swelling of the parenchymal cells which may lead to their disintegration.

Hepatocellular death (necrosis and disintegration of parenchymal cells) has been considered to be the basis of alcoholic hepatitis (77). The three essential features of alcoholic hepatitis are: spotty parenchymal cell damage, inflammation and fibrosis. In the inflammatory response polymorphonuclear leucocytes usually predominate. The inflammatory cells are found around damaged parenchymal cells. Pericellular or perisinusoidal fibrosis is found around damaged parenchymal cells. However, the histopathological features of alcoholic hepatitis are not specific to alcoholic liver disease. Inflammation and pericellular fibrosis have been found in a range of disorders such as cholestasis, Wilson's disease, obesity and vitamin A toxicity as a consequence of overdosing of retinoids (78,79).

In the course of many chronic liver diseases, the loss of parenchymal cells is accompanied by an increase in intrahepatic connective tissue, particularly collagen. As well as pericellular or perisinusoidal fibrosis, perivenular fibrosis is found in alcoholic liver disease (80,81). The progressive hepatocellular death in the perivenular region may result in perivenular fibrosis. Perivenular fibrosis progresses to scarring of the wall of the terminal and sublobular hepatic veins with gradual narrowing of their lumina. Perivenular fibrosis is often associated with perisinusoidal fibrosis (81), and these changes may represent two aspects of a similar lesion involving vascular structures of different size (82). The progression of the disease results in dissection of the liver parenchyma by developing fibrous septa, which interconnect portal and perivenular areas of liver lobules. Eventually, cirrhosis develops.

Cirrhosis is defined as the transformation of the normal lobular parenchymal architecture into regenerative nodules surrounded by fibrous septa. Regeneration nodules originate from proliferation of parenchymal cells which survived preceding hepatitis. Alcohol cirrhosis is initially micronodular, however the nodules may enlarge by compensatory hyperplasia (83). The characteristic histopathological features of cirrhosis and fibrosis are not specific for alcohol liver disease. They are also found in liver diseases of other etiology.

The contribution of parenchymal cells to hepatic collagen production and deposition in normal and diseased liver is unknown (84). Although parenchymal cells produce collagen *in vitro*, they produce 25 times less collagen (type I, III and IV) than fat-storing cells (85). This contrasts with the results of *in vivo* studies in rats. Rat parenchymal cells of normal liver (86) and of carbon tetrachloride-induced fibrotic liver (87) may have a major role in collagen production.

1.4.3 Endothelial cells

Unlike vascular endothelial cells, liver endothelial cells lack a continuous basement membrane and contain pores (fenestrae) that are arranged in so-called sieve plates,

which allow direct contact between the sinusoids and the cells that are located behind the endothelial barrier (61). The fenestrations have a diameter of about 100 nm. The cells possess numerous bristle-coated membrane invaginations. They also contain many pinocytotic vesicles, tubular structures and lysosome-like vacuoles that are indicative of a well-developed endocytic capacity (88).

It has been demonstrated that chronic ethanol consumption affects endothelial fenestration in the liver sinusoids of rats (89), baboons (90) and humans (91,92) by decreasing the number of fenestrations. Several mechanisms have been proposed to account for this alteration in the sinusoidal endothelium. Mak *et al.* have suggested that alterations in sinusoidal blood flow, intraluminal pressure, O_2 content, degree of hypoxia, or deposition of collagen and other connective tissue proteins may contribute to the defenestration of the endothelium (90). Alterations in the sinusoidal endothelium result in disturbances in the exchange of substances between parenchymal cells and sinusoidal blood, and this may contribute to the pathogenesis of alcoholic liver disease.

Endothelial cells may be involved in synthesis and degradation of connective tissue components. Several extracellular matrix-components - collagen and fibronectin - were immunohistochemically found in the rough endoplasmic reticulum of these cells in normal rat and human liver (93,94), in endothelial cells of carbon tetrachloride-treated rat liver (95), in endothelial cell cultures derived from normal rat liver (96) and from human fibrotic liver (97). However, the increase in collagen content in cirrhosis is primarily due to collagen types I and III, whereas endothelial cells probably produce type IV collagen mainly (96). The role of endothelial cells in collagen metabolism of the diseased liver is discussed in chapter 6.

1.4.4 Fat-storing cells

Fat-storing cells, also called lipocytes, stellate cells, perisinusoidal cells or Ito cells, are located in the space of Disse; the cytoplasmic processes of the cells extend between the endothelial lining of the sinusoids and the parenchymal cells. They contain variable numbers of lipid droplets, variable amounts of rough endoplasmic reticulum, relatively large Golgi apparatus and few mitochondria. In addition, fat-storing cells contain microfilament bundles, running parallel to the plasma membrane, and dense bodies; the latter serve as anchoring sites for the thin filaments. The presence of these structures suggests that fat-storing cells are contractile cells. In addition, fat-storing cells have been shown to contain the muscle-specific intermediate filament, desmin (98,99). In view of the close proximity of these cells to the endothelial lining of the sinusoids, it has been suggested that the contractility of the fat-storing cells may enhance the contraction of the sinusoids in regulating the flow of blood in the liver lobules (61).

Cell junctions between fat-storing cells and parenchymal cells have been observed in

the livers of humans and baboons (100,101). The attachment of the lipocytes to parenchymal cells indicates that the former are fixed or stationary cells. The linkage may contribute to the spatial organization of the fat-storing cells in the Disse space. Furthermore, the close proximity of the fat-storing cells to the parenchymal cells may contribute to the exchange of retinoids (see 1.6.1) between the two cell types (102). However, there is no morphological evidence for the existence of these specialized cell-cell contacts in livers of all mammalian species; they have never been documented in rat liver.

Fat-storing cells play a central role in the uptake and storage of retinoids by the liver. The involvement of fat-storing cells in vitamin A storage has been suggested by Wake (103), who reported that gold chloride staining of these cells, originally described by Kupffer in 1876, is based on the reduction of gold chloride by vitamin A. The fat droplets of these cells display a rapidly fading green fluorescence at 328 nm, which is characteristic for vitamin A (104). Based on biochemical determinations of isolated and purified fat-storing cells, the lipid droplets consisted of approximately 42% retinyl ester, 28% triglyceride, 13% cholesterol and 4% phospholipid (105). Other data has shown that these cells possess the binding proteins (cellular retinol-binding protein, cellular retinoic acid binding protein) necessary for handling free retinol and retinoic acid and contain retinyl ester hydrolase which plays an important role in the transformation of retinol into retinyl esters and vice versa (106-108). It was concluded that fat-storing cells contained the majority of the total retinoids found in the liver (63,105). The role of fat-storing cells and vitamin A in liver disease is discussed and reviewed elsewhere in this thesis (see chapter 2, 3 and 5).

Fat-storing cells have been considered to have a close association with fibrogenesis in the liver (101,109-112,1116-118). On the basis of morphological data, the hypothesis has been put forward that fat-storing cells actually represent fibroblast precursors or resting fibroblasts (101,109-112).

In normal liver, fat-storing cells show very low mitotic activity. Mitotic activity is only observed in experimental liver injuries (113,114) and after partial hepatectomy (115), but even then very rarely. In acute liver injury induced by CCl_4-treatment in rats (109,114,116) or by alcohol in humans (110,117), fat-storing cells adjacent to necrotic parenchymal cells in the perivenular zone appear to proliferate. [^{3}H]thymidine incorporation experiments showed that there is an marked increase in fat-storing cell DNA synthesis at or before the time, when an increase in the number of fibroblast-like cells was observed in the necrotic areas of the liver lobules (116).

In experimental alcoholic and CCl_4-induced liver fibrosis in baboons and rats, respectively, but also in alcoholic liver disease in humans, fat-storing cells exhibit ultrastructural changes. The progression of hepatic fibrosis is accompanied by the transformation of fat-storing cells into "transitional" cells. The name "transitional cell"

was first used by Kent *et al.* (118), to describe cells with ultrastructural features intermediate between fat-storing cells and fibroblasts, probably in transition between the two cell types. The cells were characterized as "activated" fat-storing cells, indicated by a reduction in number and size of fat droplets and by hypertrophy of the rough endoplasmic reticulum in their cytoplasm (109-111). The dilated rough endoplasmic reticulum enclosed a flocculent or fibrillar material in their channels indicative of their synthetic activity. Different studies have shown that the development of rough endoplasmic reticulum in the "transitional" cells correlates with progression of the hepatic fibrosis (101,111). The abundance of collagen fibres in the extracellular space in close proximity to the fibroblast-like cells was considered as evidence that the latter cells promote collagen deposition in the space of Disse. In addition, the rate of collagen synthesis which was measured by the method of incorporation of [^{3}H]proline into collagen, correlated with the degree of morphological change of rough endoplasmic reticulum in fat-storing cells (111).

Immunohistochemical studies (93,119) with specific anti-collagen antibodies and Northern hybridization analysis reveal that fat-storing cells and fibroblast-like cells are involved in the production of collagen type I, III and IV in normal and fibrotic liver. In normal human liver, fat-storing cells stained strongly for type IV collagen and expressed lesser amounts of type I and III collagen (93). These results were consistent with the finding that freshly isolated fat-storing cells from normal rat liver primarely contained type IV procollagen mRNA with lesser amounts of type I and III procollagen mRNA (120). However, in cirrhotic human liver all three types of collagen increase and type I collagen is markedly increased in advanced disease (93). In experimentally-induced liver fibrosis in the rat, immuno-electron microscopic observations mainly demonstrated type I and III collagens in the rough endoplasmic reticulum, Golgi apparatus and vesicles close to the cytoplasmic membrane of the fat-storing cells (119). Furthermore, fat-storing cells in culture which changed their phenotype into a fibroblast-like cell as observed in liver fibrosis, altered their collagen gene expression in a manner similar to the apparent effect of fibrogenic stimulation on these cells *in vivo* (120). In particular the collagen gene expression of type I collagen was increased; however type III and IV procollagen mRNA levels were also elevated.

The glycoproteins laminin and fibronectin are present in the extracellular matrix of the normal and fibrotic liver. Studies on the cellular source of laminin in the space of Disse of normal rat and human liver suggested that fat-storing cells, together with endothelial cells, are the major sites of production (121). Fibronectin was mainly found in parenchymal cells, however fat-storing cells also expressed low amounts (93). Increased amounts of both glycoproteins are usually found in fibrotic and cirrhotic livers, but no change in the pattern of cellular distribution of either protein was observed (93,121).

The role of fat-storing cells in liver fibrogenesis is discussed in chapters 5 and 6 of this thesis.

1.4.5 Myofibroblasts

Myofibroblasts have also been implicated as effector cells in hepatic fibrosis. These non-parenchymal cells were initially suspected because of their proximity to fibrotic regions in intact liver and furthermore they have been shown subsequently to produce extracellular matrix proteins in culture (122).

In normal liver, myofibroblasts are localized exclusively in the region of the terminal hepatic venule. They contain little or no cytoplasmic lipid. Myofibroblasts are identified ultrastructurally by convoluted nuclei and the presence of basal lamina-like material surrounding the plasma membrane (122). They increase in number following liver injury but do not extend into the normal hepatic lobule (80). In cirrhotic liver, myofibroblasts are observed in the fibrotic septa and in the region of the portal tracts together with fibroblast-like or "transitional" cells.

Whether myofibroblasts constitute a unique cell population in the liver is uncertain. Confusion arises in part because they bear some resemblance to "transitional" cells. Myofibroblast contain no intracellular vitamin A and exhibit a dilated rough endoplasmic reticulum as do transitional cells. Furthermore, each cell type contains myogenic characteristics, including cytoplasmic filaments, dense bodies and pinocytotic vesicles (80,112). Both contain the intermediate filament desmin. However, in contrast to transitional cells, myofibroblasts contain α-smooth muscle actin (123). On the basis of both morphology and their distribution in the fibrotic liver, myofibroblasts may represent another phenotype of fat-storing cells (see Chapter 6 of this thesis).

1.4.6 Kupffer cells

Kupffer cells are phagocytic cells lying in the sinusoids of the liver adjacent to the fenestrated endothelium which gives access to the parenchymal cells (124). They are in a strategic position to clear bacteria and endotoxins, viruses and immune complexes from the portal blood. They are typical macrophages which become activated to enhance their defensive role.

The cell body of a Kupffer cell shows numerous microvillous projections, blebs, lamellipodia and occasionally fillapodia. Their cytoplasm contains many well-developed heterogeneous lysosomes, pinocytotic vesicles and cisternae. The abundance of these organelles and the variety of lysosomal enzymes reflect the state of activation.

Kupffer cells endocytose large quantities of materials from the circulation. Examples of materials endocytosed by Kupffer cells include virus particles, collagen chains (125)

and endotoxin (126). Endocytosis is achieved by pinocytosis and phagocytosis. Pinocytosis of plasma occurs continuously and does not necessarily require contact of particulate or macromolecular material with the cell membrane for initiation (127). Pinocytosis of ligand bound to cell surface receptors has also been observed (128). Phagocytosis initially involves recognition of material at the cell surface and is followed by internalization (129). Recognition occurs at two levels. In the circulation it is mediated by circulating recognition factors. Circulating recognition factors can be divided into two groups: those that are non-immune-specific such as fibronectin, and those that are immune-specific such as IgM- and IgG-antibodies. At the cellular level it is mediated by cell surface receptors. Recognition at the cellular level involves particles coated with fibronectin, IgG, IgM or complement.

Pinocytosis and phagocytosis end with delivery of the contents of the vesicles and vacuoles into lysosomes. Depending on the composition of the endocytosed material, it may be degraded by lysosomal enzymes. Products of lysosomal digestion may be re-utilized or excreted. Furthermore, the process of endocytosis may result in the release of lysosomal proteolytic enzymes (130). Kupffer cells contain many enzymes such as cathepsin B, beta-acetyl-glucosaminidase and beta-glucuronidase. When released into the pericellular environment, lysosomal-derived enzymes are important adjuncts in inflammatory reactions.

Interest in the effect of alcohol on Kupffer cells has mainly focused on the known predisposition of alcoholics to infections (131). Acute and chronic alcoholic intoxication depresses Kupffer cell phagocytosis (132,133). Therefore, it seems probable that endotoxins that enter the portal vein, when the permeability of the gut mucosa is increased by alcohol intoxification, will be able to damage parenchymal cells. Once endotoxin is able to by-pass the Kupffer cell barrier it may play a key role in the development of alcohol hepatitis, which may lead to cirrhosis after a prolonged period.

Liver fibrosis may also occur secondary to the phagocytic activity of Kupffer cells. Moreover, phagocytosis may lead to the release of aggressive lysosomal enzymes which may induce hepatic cell injury. Kupffer cells are activated to produce proteins which stimulate liver cells to proliferate and to synthesize extracellular matrix proteins (62,134). Kupffer cells from rats with CCl_4-induced chronic hepatocellular injury secrete soluble, non-dialyzable factors that promote the proliferation and synthesis of collagen and proteoglycans by fat-storing cells (62,134). It has recently been observed that these factors are cytokines, such as transforming growth factor-β1, tumour necrosis factor-α and interleukines (120,135), whose production is enhanced during alcoholic liver fibrogenesis in the rat (135).

1.5 Biochemical aspects of liver fibrogenesis

This chapter presents biochemical aspects of human alcoholic liver fibrosis/cirrhosis. The matrix proteins and the role of liver cells in the development of fibrosis are emphasized.

1.5.1 The extracellular matrix of the liver

The extracellular matrix of the liver has been shown to be a dynamic component with an active metabolism and the ability to convey information to liver cells (136). It plays a crucial role in cell attachment (137), cell differentiation and filtration of nutritional components (138). It forms a fine network that interacts with cell surface components. Therefore, the extracellular matrix keeps the cells informed of the changes in the microenvironment. Furthermore, the maintenance of the differentiated functions of all the different types of liver cells is strongly dependent on the extracellular matrix.

The normal liver tissue biomatrix is composed of three main classes of macromolecules: collagens, glycoproteins and proteoglycans. The matrix is produced locally and contains components derived from the various cell types in contact with the matrix. The matrix can be of different composition and is classified into types dictated by the type of collagen used as the scaffolding. Each collagen type is associated with specific adhesion (glyco)proteins and proteoglycans.

Collagen

Collagens are the major components of the extracellular matrix of the mammalian liver. They comprise a family of proteins of at least 11 genetically-distinct types (139). Roman numerals from I to XI have been assigned to each collagen. The constituent chains are specified by the Greek letters α, β and γ to designate monomeric, dimeric and trimeric chains respectively. Most of the molecules are homopolymers made of three identical chains, but some are heteropolymers and contain two or three genetically-different α-chains; these are numbered $\alpha 1$, $\alpha 2$ and $\alpha 3$. In the liver, collagen is present around vascular structures including sinusoids. The interstitial collagens type I and III are the most abundant collagens of the liver. In rats and humans, type I accounts for 45% (140) and 40% (141) of total liver collagen, respectively. Two genetically different α-chains are present: two identical α_1(I)-chains and one α_2(I)-chain. Collagen type I is mainly localized to the vascular spaces (portal tracts and central veins) and occupies some strategic positions in the space of Disse; it is observed at points of branching or inflection and in the scaffold of the hepatic lobule (138). Type III collagen accounts for approximately 40% of total liver collagen (140,141). It consists of three identical α_1(III)-chains. It is present in vascular spaces but, unlike type I collagen, it is associated with processes of fat-storing cells in the space of Disse (94). Type IV collagen is only a minor

component of the total liver collagen and is mainly localized to vascular, lymphatic and canalicular structures which contain continuous basement membranes (138). In the perisinusoidal space, it is localized in discontinuous sheets of basement membrane-like material. The absence of a structurally-defined basement membrane facilitates exchange between blood and parenchymal cells. Two genetically-different α-chains have been indicated: α1(IV) and α2(IV). Three different molecular species have been recognized: $[\alpha1(IV)]_2\alpha2(IV)$, $\alpha1(IV)_3$ and $\alpha2(IV)_3$.

The major interstitial collagen polypeptides of the liver (type I and III) are synthesized as precursors on membrane-bound ribosomes of the rough endoplasmic reticulum (RER) of various liver cells (see 1.4). These precursors, called pro-α-chains, have a "signal peptide" required for "threading" secretion proteins through the membrane of the RER. Furthermore, they contain three distinct domains: an NH_2 domain, a COOH domain, both called extension peptides, and a 300-nm-long helical domain. The extension peptides guide the assembly of collagen in the cells and prevent the intracellular formation of large collagen fibrils. During the process of secretion, the extension peptides of types I and III procollagen α-chains are cleaved by procollagen peptidases, converting procollagen chains into collagen chains. In contrast to type I procollagen, type III procollagen is not processed completely (142). Type III collagen of the liver is deposited as procollagen containing its NH_2 domain. Antibodies directed against this antigen permit both intra- and extracellular immunolocalisation of procollagen type III. The triple helix extending over a major part of the molecule is characteristic of the interstitial collagen molecules. This consists of glycine, present at every third amino acid, and proline and hydroxyproline which are required to form and stabilise the triple helix. In the extracellular space, the collagen α-chains spontaneously assemble into fibrils under the direction of electrostatic and hydrophobic interactions between the repeated GLY-X-Y sequences of the helical domains.

Type IV procollagen undergoes little, if any, processing (139). Biochemical data suggest that there are almost no chemical or structural differences between procollagen and collagen molecules (143). Unlike type I and III collagen, type IV collagen molecules are flexible thread-like molecules terminating at the -COOH end of the protein in a globular knob (NC1 domain) and at the $-NH_2$ end in a compact helical zone (7S domain). They contain a relatively large region of nonhelicity (NC2) domain adjacent to the compact helical zone. The overall length of the monomers, as visualized by rotary shadowing (144), is approximately 400 nm. The aggregation of type IV molecules has been modelled on the basis of these observations. The basic unit is a tetramer formed by the association of four molecules at their compact helical ends while larger units are constructed by the association of tetramers, by virtue of interaction sites in the globular region at ends of the tetramer arms. This arrangement, which is formed largely through end-to-end associations of the molecules, gives rise to an open mesh-like network, which

serves as a scaffold for sheaths of basement membrane which function, at least in part, as selective filtration barriers.

Collagen fibrils of type I and III in the extracellular matrix of the liver are greatly strengthened by the formation of di-, tri- and tetrafunctional covalent intra- and intermolecular cross-links between the constituent collagen molecules. Two pathways for crosslinking have been recognized for the interstitial collagens; one based on the lysine aldehyde pathway, the other on the hydroxylysine pathway (145). Which pathway operates seems to depend on whether lysine residues in the non-helical region of the α-chains (extension peptides) are hydroxylated or not at this site, where both will later be oxidatively deaminated by the enzyme lysyl oxidase. The resulting lysine- and hydroxylysine aldehydes are involved in the formation of cross-links. These borohydride-reducible cross-links have also been shown to be precursors of more complex multivalent intermolecular bonds which are formed spontaneously during maturation and ageing of collagen. It has been proposed that the increased stability of mature collagen is due to the presence of these multivalent cross-links, forming a system of lateral and transverse cross-links in the fibre. The chemical structure of the mature cross-links is unknown for the lysine aldehyde pathway, but the final products in the hydroxylysine aldehyde pathway have been identified for many tissues. (145-147). The appearance of these non-reducible cross-links is gradually linked to the disappearance of the reducible cross-links during maturation, suggesting that the latter are intermediates. Based on these results, biochemical methods have been developed to determine the maturity of the collagen (see Chapter 7).

Glycoproteins

Most liver cells require attachment to a substrate for survival, growth and differentiation. Such a substrate is provided by the glycoproteins of the extracellular matrix which are synthesized by the cells themselves. Fibronectin and laminin are the main adhesion glycoproteins: a generic term for molecules that attach cells to collagen. They play an important role in the migration of cells through the tissues and determine whether a cell will, or will not, be immobilized.

The glycoproteins have well-defined domains that interact with cell surface receptor molecules and collagen scaffoldings (148). Fibronectin and laminin contain a cell attachment site which is a common structure in many, perhaps all, of the adhesive proteins in extracellular matrices (149,150). The amino acid sequence arginine-glycine-aspartic acid promotes the cell attachment activity of these glycoproteins. Both proteins show a certain degree of specificity for collagen; fibronectin is associated with collagen type I and III (151,152) and laminin is mainly associated with type IV collagen (153). As a consequence of this association, fibronectin is mainly present in vascular spaces and in the space of Disse of the normal liver (137,153,154). Laminin is mainly localized to

the endothelial lining of hepatic arteries, portal veins, lymphatic vessels and canalicular structures which contain basement membranes. It is generally absent from the sinusoidal space, but there may be some stretches of laminin in discontinuous sheets of basement membrane-like material (153,154).

Proteoglycans

The liver contains small amounts of proteoglycans. Proteoglycans are composed of a core-protein to which glycosaminoglycans are covalently bound. The glycosaminoglycans are sugar polymers composed of alternating glucoronic acid and sulphated hexosamine residues. The major proteoglycans in the liver are heparan sulphate and heparin proteoglycans (155). Both are associated with the plasma membrane of the parenchymal cells and both are polymers of glucoronic acid and glucosamine. Only a minority of the proteoglycans are bound to hyaluronic acid and these may form very large aggregates of proteoglycans in the extracellular matrix.

Heparan sulphate and heparin proteoglycans form complexes with fibronectin and laminin. Both are mainly associated with type III and type IV collagens (156). Other proteoglycans of the liver, like dermatan sulphate and chondroitin sulphate, are mainly associated with fibronectin and type I collagen; this matrix type is produced by fibroblasts in the vascular structures of the liver.

Proteoglycans can modulate the adhesive properties of other extracellular matrix proteins and can affect the fibrillogenesis of collagen (157,158). Various soluble proteoglycans, when included in cell attachment assays in which cells are attached to fibronectin or type I collagen, can inhibit cell attachment (159). The mechanism of this inhibition appears to be that the proteoglycans bind to adhesion proteins (fibronectin, laminin) or collagen and cover up or change the attachment sites with which the cell would interact. Furthermore, proteoglycans may interfere with the fibrillogenesis of collagen. *In vitro* studies have shown that, depending on the composition of the proteoglycans, fibril formation of collagen can be inhibited or accelerated (157). These results indicate that proteoglycans play a role in the formation of the extracellular matrix and may regulate the permeability, stability and strength of the matrix between the cells.

1.5.2 The extracellular matrix in alcoholic liver fibrosis

The hepatic extracellular matrix plays a major role in the function of the liver in its normal state. Its alteration is a prominent feature of alcoholic liver disease and may be directly responsible for deranged hepatic function.

The composition of the extracellular matrix alters in all kinds of chronic liver disease. It is not only the composition of the matrix between the liver cells which is changed, but also the site of deposition is altered. Furthermore, the synthesis/break-down ratio of

various components of the matrix in fibrotic and cirrhotic livers is increased.

Collagen content of fibrotic liver

Increased collagen deposition is a crucial factor in the transition from fatty liver to alcoholic liver fibrosis; in general, all collagen types are produced in larger quantities. Quantitative studies of liver collagen content in alcohol-induced human cirrhotic livers showed a 4- to 7-fold increase when compared with normal human livers (160,161).

Liver collagen content is measured by colorimetric hydroxyproline determination (162,163). Hydroxyproline is an amino acid which is rarely observed in proteins other than collagen. The collagen content of the liver is expressed as the amount of hydroxyproline per unit weight of liver tissue (see Chapters 2-5).

Qualitative alterations in the types of collagen have been described in liver fibrogenesis (93,153,164). In alcoholic liver cirrhosis, type I collagen predominates, and the ratio of type I to type III collagen is greater than 1. However, the increase in the ratio of type I to type III is not observed in all human cirrhotic livers. Human liver containing less than 20 mg of collagen per gram of fresh liver tissue has a normal ratio of type I to type III collagen. The significance of the increased ratio is not well understood. However, it has been suggested that while the relative proportion of liver collagens is maintained within normal limits, the fibrotic process may be reversible. When the ratio of type I to type III is greater than 1, the liver may have reached a "committed stage", and fibrogenesis will then proceed to cirrhosis.

Distribution of collagens, glycoproteins and proteoglycans.

The distribution of collagens, fibronectins and laminin in the alcoholic cirrhotic liver has been investigated using fluorescence and peroxidase-labelled antibodies. The most striking finding is that the normal distribution pattern is lost: collagen types I, III and IV as well as the noncollagenous glycoproteins (laminin and fibronectin) are found in fibrous septa that surround the nodules of parenchymal cells (165). Intralobular or intranodular, type I and III collagen are present in greater amounts than in normal liver but they still have a heterogenous distribution in the sinusoids. Type IV and laminin are distributed continuously along the sinusoids (166). Changes in the distribution pattern of various collagens and glycoproteins in the fibrotic liver are reported and discussed in Chapter 6.

There are no reliable data on the total concentration and distribution of proteoglycans in the cirrhotic liver. Although changes in their composition have been reported, previous methods used for the quantification of proteoglycans did not provide reliable and precise information.

1.6 Role of nutrition in the pathogenesis of alcoholic liver disease

The pathogenesis of alcohol-related liver disease (ALD) appears to be complex. Both the amount and the duration of alcohol intake are important factors (167). Although a clear relationship exists between alcohol consumption and alcoholic hepatitis and cirrhosis, total alcohol consumption alone (related to the direct hepatotoxic effects of ethanol itself) does not explain why only 17-30% of heavy drinkers develop liver injury (167,168). For this reason, other factors such as genetic background, the quality of the immune response (169), but also nutrition, have been investigated.

The role of nutritional factors in the pathogenesis of alcoholic liver disease has received much attention but remains controversial. Before 1960, liver disease in heavy drinkers was thought to be due primarely to protein malnutrition (170). This was based on animal experiments which showed that ethanol administered in the drinking water of rats did not produce liver damage unless accompanied by nutritional deficiencies (170,171). Epidemiologic studies reporting a reduced intake of protein and other essential nutrients in alcoholics with liver disease also supported the nutritional hypothesis of liver damage (172).

Direct toxic effects of alcohol on the liver have been reported in controlled studies with human volunteers (173,174). Morphologically and biochemically, an increase in hepatic lipid was demonstrated when ethanol was given either as a supplement or as an isocaloric substitution for carbohydrate together with an otherwise nutritionally-adequate diet. Fatty liver was observed, even with a high-protein, vitamin-supplemented diet, and was accompanied by ultrastructural changes in mitochondria and by elevations of hepatic transaminases in blood (11,173). However, the later stages of alcoholic liver disease, such as fibrosis or cirrhosis were not observed in these human studies.

In summary, alcoholic liver disease cannot be explained as an exclusive effect of malnutrition, nor can a direct effect of alcohol alone explain all stages of alcoholic liver disease. The daily amount and duration of alcohol consumption are important in the etiology of liver disease, but nutritional factors may play a role in modulating the hepatotoxicity of ethanol. For instance, the incidence of malnutrition in alcoholics is so high that it may be impossible to identify the contribution of each factor (175). Furthermore, chronic alcohol consumption may contribute to malnutrition despite adequate diet intake. Factors such as ethanol-induced malabsorption from the intestines (176), inhibition of protein synthesis by alcohol and acetaldehyde (177), and increased urinary excretion of nitrogen (178), may contribute indirectly to malnutrition, which may modulate the hepatotoxicity of ethanol.

1.6.1 Vitamin A

The liver is the most important organ in the homeostasis and metabolism of retinoids. Parenchymal cells are involved in the uptake of retinoid-containing chylomicron remnants from the blood (179) and in the processing of retinoids by the endoplasmic reticulum (180). The parenchymal cells are the site of synthesis of the retinol-binding protein, which is secreted into the blood when it has bound retinol (181,182). Retinol bound to the retinol-binding protein is transported to the organs and tissues. The mechanism for the transfer of retinoid between parenchymal cells and fat-storing cells and vice versa is not clear (see 1.4.4, 173).

The liver stores more than 95% of the retinoids present in the body, predominantly in the fat-storing cells (see 1.4.4). The amount of vitamin A stored in the liver varies greatly depending on nutritional intake. Liver and plasma levels are not correlated. Plasma levels remain fairly constant for a given individual under normal conditions. Abnormal plasma levels were only found when liver content is critically depleted or excessively expanded in the hypo- and hypervitaminosis range, respectively (184).

The hepatotoxicity of hypervitaminosis A has been well documented (79). The clinical manifestation of hepatic involvement is hepatomegaly (185); cases of portal hypertension with ascites (79) and cirrhosis (186) have been reported. Portal hypertension with ascites has been observed in the absence of cirrhosis and has been attributed to obstruction of sinusoids and terminal veins by the greatly increased number and size of perisinusoidal fat-storing cells (79).

Alcoholics have an unusual susceptibility to the hepatotoxic effects of hypervitaminosis A (187). Clinically, hypervitaminosis A and alcoholism can occur together. At present it is not possible to distinguish between the toxic effects induced by vitamin A or ethanol. Increased serum activities of enzymes such as aminotransferases may reflect hepatotoxicity but, in alcoholics, differentiating between an enzyme increase resulting from vitamin A toxicity or from direct hepatotoxic effects of ethanol is not possible.

Some toxicity may possibly be caused not only by vitamin A itself, but by some of its metabolites, the production of which might be increased by chronic ethanol intake. As mentioned before (see 1.3), some degradative pathways have been described in hepatic microsomes. Alcohol induced microsomal enzyme activity potentially may stimulate production of some toxic metabolites.

Very low nutritional intake of vitamin A may result in a low hepatic level. Low intake in combination with chronic alcohol abuse, which increases the turn-over of retinoids (188), may eventually induce hypovitaminosis A. In turn, hypovitaminosis A may affect the normal functions of the various hepatic cells of the lobule or acinus. Lieber and coworkers have suggested that vitamin A depletion is probably an important factor

contributing to the development of the lesions of alcoholic liver disease, particularly to fibrogenesis (189). This hypothesis is also supported by experimental carbon tetrachloride-induced liver injury in rats, showing that developing fibrosis is suppressed by vitamin A supplementation (190).

The role of vitamin A in liver disease is very complex and has been an important issue of our studies. It will be discussed and reviewed, both in relation to alcoholic liver disease and to carbon tetrachloride-induced liver injury (see Chapters 2 and 5).

1.7 Animal models to study the pathogenesis of alcoholic liver disease

Nowadays, the main features of human alcoholic liver injury - fatty liver, hepatitis and fibrosis, cirrhosis - are known, including their clinical, functional and morphological aspects. However, the mechanisms involves in the different forms of liver disease in alcoholics are less clear. To improve our knowledge of human alcoholic liver disease animal models are required. Extensive efforts have been made to establish a suitable experimental animal model which replicates the whole spectrum of alcoholic liver injury in humans.

In first attempts, rats were administered ethanol as part of their drinking water. Rats, however, have a natural aversion to ethanol, which resulted in a low intake. As a consequence, blood ethanol levels were too low to cause significant liver damage when the diet was adequate (171). A combination of ethanol administration and a diet deficient in both protein and lipotropic factors (choline and methionine) leads to a fatty liver and cirrhosis (171) but, nowadays, it is generally believed that this model has little, if any, relevance to human alcoholic liver injury. Humans are far less susceptible to protein and lipotropic factors than rodents since human liver, unlike rat liver, contains very little choline oxidase activity (191). Furthermore, treatment with choline of patients suffering from alcoholic liver injury has been found to be ineffective because of continued alcohol abuse (192). Massive supplementation with choline failed to prevent the fatty liver produced by alcohol in volunteers (193).

In 1963, Lieber and coworkers reported that rats fed 36% of calories as ethanol with a so-called nutritionally-adequate diet, developed fatty liver (194,195). The experiments were based on a feeding technique that overcomes the natural aversion of rodents to alcohol because ethanol was incorporated in a nutritionally-adequate liquid diet which forces experimental animals to ingest ethanol. The diet contained 35% fat, 18% protein, 11% carbohydrate and 36% ethanol as percentages of total calorie intake (196). A control liquid diet substituted isocaloric carbohydrate for ethanol. Rats reached significantly higher blood ethanol levels with the ethanol-containing diet than could be achieved through the drinking water technique. However, rats fed the ethanol-containing

diet only developed fatty liver; progression to hepatitis, fibrosis and cirrhosis has never been reported using the ethanol-containing diets of Lieber-DeCarli (196). In addition, the adequacy of the Lieber-DeCarli diet in terms of overall nutrition has to be doubted (chapter 2); for instance, if the fat content of the ethanol-containing liquid diet of rats is decreased from 35 to 25%, hepatic tryglyceride accumulation significantly decreases (197). Furthermore, the chain length of the dietary fatty acids is important. Medium-chain fatty acids reduced the capacity of ethanol to produce a fatty liver in rats as compared with long-chain fatty acids (198). These results indicated that the degree of fatty liver in rats cannot be explained as an exclusive effect of ethanol, but that the composition of the liquid diet also plays a modulating role for the etiology of fatty liver.

In 1983, Lieber and coworkers reported a new rat model to study alcoholic liver disease (199). This model was based on the possible toxic interference of vitamin A metabolism with ethanol metabolism in the liver (see 1.3). High doses of vitamin A were added to the ethanol-containing liquid diets which were composed as described above. Fatty liver, necrosis of the liver parenchyma and centrilobular fibrosis were observed in rats fed the ethanol-containing diet for 9 months. This model further indicated the modulating role of nutrition in alcoholic liver disease and will be discussed in detail in chapter 2.

Further approaches have been made to determine the pathogenesis of liver fibrosis and cirrhosis using the rodent model. French and coworkers developed a rat model of alcoholic liver disease which was based on an intragastric feeding technique of an ethanol-containing liquid diet (200). Pair-fed controls consumed a liquid diet containing isocaloric carbohydrates for ethanol. High blood ethanol levels were obtained with the ethanol-containing diet and fatty liver followed by centrilobular necrosis and fibrosis were reported over a period of 6 months. However, the severity of alcoholic liver disease induced in this model was nutrition-dependent. With this model, French and coworkers showed that a low fat (5% of calories) diet causes fatty liver and necrosis of liver parenchyma, but no fibrosis in the liver (201). A high fat (37% of calories) and low protein (14% of calories) diet, including 29% and 20% of the calories as ethanol and dextrose, respectively, also induced fatty liver and necrosis of liver parenchyma and liver fibrosis was also observed (202).

Nakaysukasa and coworkers attempted to elucidate the different effects of various alcoholic beverages, such as sake and whisky, and ethanol on liver fibrogenesis in the rat (203). Ethanol and the beverages were included in a liquid diet containing 15% carbohydrate, 35% fat, 15% protein and 35% of the various alcoholic beverages as percentage of total calories. After 4 months of the liquid diet administration, sake induced hepatic collagen accumulation, particularly around central veins and in a pericellular distribution. Necrosis of liver parenchyma was not observed. Under similar experimental conditions, whisky and ethanol did not produce any changes.

Thus, for the study of alcoholic liver disease in the rat, long-term administration of a ethanol-containing, high fat/low protein liquid diet is necessary. The percentage energy intake as ethanol has to be 30-35% of total calories consumed. Malnutrition cannot be excluded in these alcoholic liver disease models in the rat. Micronutrients, such as vitamin A, have been shown to interfere with the pathogenesis of alcoholic liver disease.

The ethanol-containing liquid feeding technique was extended to primates such as baboons (204,205). These animals were fed a total diet in which ethanol represented 50% of the total calories consumed. The entire spectrum of alcoholic liver injury was initially reported to occur in these animals which were fed the diet for 9 months to 4 years (204,205). However, it was subsequently noted that the morphological features were similar to, but not identical to that in alcoholic patients; in the baboons, there was swelling and ballooning of the parenchymal cells but the inflammatory infiltrate consisted mainly of mononuclear cells (206). Furthermore, the cytoplasm of the parenchymal cells did not contain alcoholic hyalin. In experiments with other monkeys, administration of ethanol as 50% of calories for 4 years only resulted in fatty liver (207).

Although the (sub)primate animal models show some similarities with the liver lesions of human alcoholic liver disease, it still has major disadvantages which make these models virtually useless for studing alcoholic liver disease. First of all, the use of baboons or other (sub)primates in experimental research is reserved to a few research groups in the entire world, which are allowed to or can afford the use of such animals. The results reported cannot be checked or reproduced, which reduces the scientific value of the models. Furthermore, it takes a very long time before liver fibrosis or cirrhosis, if ever, are induced; for instance, of the original baboons studied by Lieber and coworkers, only 6 developed cirrhosis whereas 12 did not even after 6 years of ethanol-containing diet administration (206). Scientific progress with respect to detection of cells and mediators involved in the process of liver fibrosis and cirrhosis may be inhibited by such long-term ethanol models. Therefore, much effort has been put into alternative experimental animal models which more closely resemble the pathogenesis of liver fibrosis and cirrhosis in human alcoholic liver disease, but require only a short induction time. In the search for an alternative, adequate model of human alcoholic fibrosis and cirrhosis, the carbon tetrachloride (CCl_4) model has been shown to be less time-consuming and has helped in our understanding of pathogenic mechanisms of liver fibrosis and cirrhosis in human alcoholic liver disease (208).

In 1936, it was reported that repeated administration of CCl_4 to rats results in cirrhosis of the liver (209). Since that time, CCl_4-induced fibrosis and cirrhosis have been the subject of many studies using rats, dogs, mice, pigs, rabbits, hamsters and guinea pigs. CCl_4 may be given by subcutaneous, intramuscular or intraperitoneal injections, may be administered through a gastric tube, or may be administered by inhalation in a closed chamber. A single dose results in acute liver damage which has been widely employed

to study the morphological and biochemical features of cellular lesions, particular necrosis and fatty liver. To produce experimental fibrosis and cirrhosis, it is necessary to give repeated doses of CCl_4 regardless of the animal species and route of administration; furthermore, the time interval between each insult should not be too long, since damaged cells can recover, rendering the effect of the toxin non-cumulative. In our laboratory, extensive fibrosis in rats is usually present after 4 weeks of treatment, cirrhosis usually after 8 weeks of treatment.

The hepatotoxic effects of CCl_4 are dependent upon its metabolic activation in the endoplasmic reticulum of the parenchymal cell to reactive intermediates (210). The metabolic activation is associated with the NADPH-cytochrome-P450 electron-transport chain. A major effect of the CCl_4 intoxification is the production of free-radicals, which induce lipid peroxidation and covalently bind to cellular proteins.

In experimental CCl_4-induced fibrosis and cirrhosis, the major features of human alcoholic liver injury are reproduced (211). The production of CCl_4-induced cirrhosis involves progressively repeated episodes of focal centrilobular parenchymal cell necrosis, fibrosis resulting from collapse of previously-existing collagen framework and *de novo* collagen synthesis and regeneration of surviving liver cells, forming nodules within connective tissue septa (212). Furthermore, in the cirrhotic animals there are signs of portal hypertension and portocaval anastomosis develop within the connective tissue septa (213).

Although there are some similarities between CCl_4-induced liver cirrhosis and human alcoholic liver cirrhosis, there are too many differences to allow a conclusion to be reached regarding the adequacy of the experimental model as a copy of the human disease. For instance, the source and structure of the stimulus for alcoholic fibrosis and its relation to liver cell necrosis are not known. Furthermore, the cellular sources of excess collagen and other matrix proteins are unknown in both the experimental model and human disease. Nevertheless, the CCl_4-model has been widely accepted as a model for the study of liver fibrosis.

AIM OF THE STUDY

Alcoholism is a major health problem in the Western world. In spite of this, little is known about the determinants of the adverse effect of alcohol on the liver. Many questions still remain to be answered regarding the pathophysiological mechanisms which lead to alcoholic liver disease.

The aim of this thesis was to study the various (histo)pathological and biochemical features of human alcoholic liver disease (such as fatty liver, alcoholic hepatitis, fibrosis and cirrhosis) in rat models. The role of nutritional factors as well the liver cells involved

in alcoholic liver disease were examined.

Chapter 2 describes the histopathological (2A) and biochemical findings (2B) of chronic ethanol consumption in combination with a high vitamin A intake in rats. Dietary retinoid is discussed in the context of its being a pathogenetic factor involved in alcoholic liver disease.

In Chapter 3 the role of nutritional factors in the pathogenesis of alcoholic liver disease is investigated. In an attempt to reproduce the various histological features of human alcoholic liver disease in a rat model, the nutritinal composition of the diet has been manipulated to resemble the life style diets of chronic alcoholics.

Chapter 4 reports on the enhancement of CCl_4 induced liver fibrosis in rats by ethanol. The aim of this study was to determine whether histological liver lesions which are considered to be characteristic of human alcoholic liver disease could be elicited in a rat model by alcohol administration.

In chapter 5 the role of dietary retinoids and carotenoids on the development of liver fibrosis is investigated. Chapter 5A shows that orally-administered retinoids do enhance or decrease CCl_4-induced liver damage, dependent on the time of administration of this vitamin relative to the treatment with CCl_4. In Chapter 5B the role of intrahepatic retinol on the induction of hepatic fibrosis by CCl_4 in rats was investigated. Results of this study support the hypothesis that intrahepatic retinoids suppress the induction of hepatic fibrosis. Chapter 5C reports on the effects of the carotenoid β-carotene on liver damage and the development of hepatic fibrosis induced by CCl_4 in rats. The aim of this study was to determine whether β carotene has a therapeutic potential on developing liver fibrosis.

Chapter 6 reports on the liver cells involved in the initial phase of CCl_4-induced hepatic fibrosis in rats. The aim of this study was to detect whether fibroblastic cells that are considered to be involved in liver fibrogenesis are decended from fat-storing cells or myofibroblasts.

Chapter 7 reports the effects of alcohol and nutrition on ageing. The aim of the study was to investigate the consequences of chronic ethanol consumption and long-term calorie restriction on age-related changes in physico-chemical properties of collagen and survival.

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CHAPTER 2

CHRONIC ADMINISTRATION OF ETHANOL WITH HIGH VITAMIN A SUPPLEMENTATION IN A LIQUID DIET TO RATS DOES NOT CAUSE LIVER FIBROSIS

A. MORPHOLOGICAL OBSERVATIONS

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B. BIOCHEMICAL OBSERVATIONS

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A. MORPHOLOGICAL OBSERVATIONS

ABSTRACT

Rats of 2 strains (BN/BiRij and WAG/Rij) were fed the ethanol-containing Lieber-De Carli liquid diet supplemented with high amounts of vitamin A for 16 months. In contrast to Lieber and coworkers, who showed liver fibrosis developing within 9 months on the same diet in Sprague Dawley rats, we were unable to demonstrate a histological and biochemical increase in liver collagen in either strain. Steatosis was present to a varying degree in both strains in ethanol-treated rats, but also in control animals. Considerable liver inflammation with focal necrosis accompanied by severe systemic inflammation was observed in 60% of the ethanol-treated WAG rats. This suggests that, at least in rats, the main effects of chronic ethanol consumption on the liver may be secondary to interference with host resistance to infections. The ethanol-high vitamin A Lieber-De Carli liquid diet does not necessarily elicit fibrosis or other characteristic histological abnormalities of human alcoholic liver disease.

INTRODUCTION

Alcoholism is a major health problem in modern Western society (1), with a heavy social and economic impact. In spite of this, very little is known about the determinants of the adverse effect of ethanol on the liver (2). Attempts have been made to develop an animal model as an analogue of human alcoholic liver disease (ALD) for investigations on the pathogenetic factors involved in alcoholic liver fibrosis and, in particular, on the mechanisms involved in the early events of ALD (3-5).

In 1983, Lieber and co-workers described a rat model to study ALD, which was based on toxicity of dietary vitamin A in combination with chronic ethanol consumption (5). Ethanol and high doses of vitamin A were incorporated in a well-defined semi-synthetic liquid diet which results in the ingestion of high amounts of ethanol, at a fixed percentage of total calories. They showed that liver fibrosis could be elicited in 9 months in all rats. However, the highly characteristic histological picture of alcoholic hepatitis in human liver, composed of focal parenchymal cell damage with swelling and polymorphonuclear cell infiltration, was not observed (5).

By using the same experimental approach, in two other rat strains than those used by Lieber and co-workers we further investigated the reliability of the model for the study of ALD. We used young adult rats to exclude the possible deleterious effects of

high doses of ethanol and vitamin A on maturation, which usually are excluded in human alcoholics. The histopathological findings in the liver were related to pathological alterations of the lung and the kidney. This report focuses on the histopathological findings. The biochemical results are presented in the accompanying paper (6).

MATERIALS AND METHODS

Animals

Male rats of the BN/BiRij and the Wag/Rij strain, aged 3 months, weight 268 ± 19 g (n = 20) and 246 ± 13 g (n = 20), respectively, were obtained from the Institute's colony (TNO, Rijswijk, The Netherlands). All rats were housed individually. A liquid diet (Bio-Servo Inc., Frenchtown, New Jersey, USA) as the only source of food and water was given. The composition of the diet was the same as used by Leo and Lieber (5). It is listed in detail in a 1989 review (7, "all purpose diet"). Briefly, the diet contained 36% of the total caloric amount either as ethanol or as isocaloric carbohydrates (dextrin-maltose). Vitamin A, as retinyl acetate, was added at a final concentration of 28.000 IU/liter, the same proportion administered to rats of the high vitamin A group reported by Lieber and coworkers (5). For each *ad libitum* ethanol-fed animal there was a paired carbohydrate-fed control in both strains. Body weight was measured every week. The rats were killed after 16 months of liquid diet administration, at the age of 19 months. Blood ethanol levels were determined with a commercially available test combination (Boehringer Mannheim, FRG) on heparinized blood collected from the tail vein in the morning (9 a.m.) and in the evening (9 p.m.).

Surgical liver biopsies

Under halothane anaesthesia, surgical liver biopsies were taken at 6, 9 and 12 months of liquid diet administration. Sections of 20 to 30 mg of the median lobe were removed with an iris scissor (Stöpler, Utrecht, The Netherlands) and fixed in 4% buffered formalin for histological examination. Bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, The Netherlands). The peritoneum and the abdominal wall were sutured with absorbable surgical suture (4-0 Dexon plus, Davis and Geck, Danbury CT, USA). The skin was closed with wound clips (Clay Adams, Parsippany, NJ, USA) which were removed one week after surgery.

Organ preparation and histopathology

The animals were fasted overnight before being killed. At the time of death, the right lung was removed and frozen in liquid nitrogen for biochemical assays. Liver tissue was collected after perfusion with phosphate-buffered saline (PBS, pH 7.4) through the portal

vein for 5 min at 5 ml/min, followed by perfusion fixation. The median lobe was excluded from fixation and frozen in liquid nitrogen for biochemical analysis. The right lateral and caudate lobe of the liver were fixed for electron microscopy using 2.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 8 min). The remainder of the liver was fixed for light microscopy in neutral-buffered formalin. A routine post mortem examination was carried out and at least kidney, left lung, heart, spleen and brain were fixed in neutral-buffered formalin for light microscopy. Tissues were routinely embedded in paraplast and sectioned at four μm thickness. The sections were stained with haematoxylin-phloxin-saffron (HPS). Liver was also stained with PAS, with and without diastase pretreatment, Elastica Van Gieson and Sirius-red F3BA (8) for connective tissue and collagen, respectively.

Light microscopy of the liver

Morphology assessment was performed by a pathologist who had been blinded to the source of the specimens. Sections of liver biopsies and of post mortem liver tissue were analyzed for the presence of steatosis, inflammation, fibrosis of the central vein (perivenular fibrosis) and of the adjacent parenchyma (zone 3), the presence of megamitochondria and of portal inflammation. Steatosis and parenchymal inflammation were graded from 0 to '+ + +'. Gradings '+', '+ +' and '+ + +' steatosis reflected an estimated involvement of 1/3, 2/3 and all of the liver parenchymal cells, respectively. Predominance of the steatosis as micro- or macrovesicular change was noted. Inflammation grade '+' reflected the presence of scattered isolated inflammatory cells in the parenchyma, '+ +' focal collection of inflammatory cells, and '+ + +' diffuse parenchymal inflammation. Predominance of round cell or polymorphonuclear cell infiltration was noted. Intermediate grading of steatosis and parenchymal inflammation was applied when necessary.

Electron microscopy and morphometric analysis

For electron microscopy and morphometric analysis, representative samples were taken from the right lateral and the caudate lobe. Samples were fixed in glutaraldehyde and postfixed with osmium tetroxide, dehydrated in graded concentrations of ethanol and embedded in epon LX 112 (Ladd Research Industry Inc., Burlington, VT, USA). Ultrathin fibratome sections were mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined under an EM 410 electron microscope (Philips, Eindhoven, The Netherlands). These sections were used to determine the relative numbers of fat storing cells (FSC) and parenchymal cells (PC). 150-200 parenchymal cells were determined per animal and only the cells with nuclei included in the plane of the section were counted. The number of FSC was expressed per 1000 parenchymal cells (Ito cell index). For morphometric analysis of the various subcellular

compartments in the FSC, photographs were taken of cells which showed a portion of the nucleus (at 10,750 x final magnification). The total cell body and the areas occupied by lipid droplets, nucleus, mitochondria and rough endoplasmic reticulum (RER) were determined using a digitiser tablet connected with a microcomputer system (MOP-Videoplan) with Kontron software (9). For each rat, 10 photographs were analyzed.

Hydroxyproline determination

300 to 500 mg of the frozen median lobe of the liver was lyophilized overnight, powdered and extracted twice with 20 ml diisopropylether to remove vitamin A (which suppresses the colour development of the assay). Traces of diisopropylether were evaporated in a waterbath at 37°C. Hydrolysis was performed by addition of 5 ml of 6 N HCL per 300 mg "wet" tissue and incubation at 120°C for 16 h in screw-capped plastic tubes (Falcon 2070). After cooling, the hydrolysate was filtered through a 0.22 μm Millipore filter. Each tube and filter were rinsed with the same volume of 6 N HCL, which was added to the first filtrate. The hydrolysates were stored at -20°C.

Hydroxyproline determination was performed using a modification of the method of Woessner (10), as described earlier (9). Briefly, a 100 μl sample of the hydrolysate was added to a mixture of 100 μl 6 N NaOH and 1.8 ml 0.3 M NaCl. One ml of 0.05 M chloramine T in citrate-acetate buffer (pH 6.0) in the presence of 1.32 M n-propanol was added to this mixture. The solution was mixed and incubated for 20 min at room temperature. The incubation was stopped by adding 1 ml perchloric acid/4-dimethylaminobenzaldehyde solution in n-propanol. Colour was developed by a 15 min incubation at 60°C. After cooling, absorbance was determined at 550 nm with an Ultraspec 4050 spectrophotometer (LKB, Bromma, Sweden). Hydroxyproline content was calculated on the basis of calibration curves using purified hydroxyproline as the standard.

Statistics

Results are expressed as the mean ± S.E.M. Statistical analysis was performed by the Student t-test for paired observations (significance level 5%, 1% and 0.1%).

RESULTS

Dietary and ethanol intake, and blood ethanol levels

The daily intake of liquid diet per kg body weight showed very little variation within each strain, but was slightly higher for BN rats (Table 1). The growth curves during the treatment period were very similar for ethanol-treated and control rats of both strains (Table 1). A gradual increase in body weight was noted in all rats.

Table 1. Body weight, dietary and ethanol intake, and blood ethanol levels of BN and WAG rats kept on liquid diet with or without ethanol.

	BN rats		WAG rats	
	control	ethanol	control	ethanol
Body weight (g)				
0 months on diet	271 ± 3	264 ± 7	248 ± 4	243 ± 4
2 months " "	320 ± 8	315 ± 11	317 ± 2	315 ± 6
9 months " "	382 ± 7	386 ± 12	382 ± 5	379 ± 7
16 months " "	434 ± 11	423 ± 19	428 ± 7	426 ± 11
Dietary intake (ml/kg body weight/day)[a]				
2 months	-	180 ± 3	-	175 ± 2
9 months	-	181 ± 6	-	171 ± 6
16 months	-	190 ± 4	-	169 ± 3
Dietary intake (ml)[a]				
2 months	-	4239 ± 126	-	3828 ± 231
9 months	-	18194 ± 474	-	16258 ± 677
16 months	-	35929 ± 1196	-	33507 ± 1262
Ethanol intake (g/kg body weight/day)				
2 months	0	9.0 ± 0.2	0	8.8 ± 0.1
9 months	0	9.1 ± 0.3	0	8.6 ± 0.3
16 months	0	9.5 ± 0.2	0	8.5 ± 0.2
Blood ethanol levels (mg/dl)				
16 months (9 am)	0	33 ± 11	0	36 ± 9
16 months (9 pm)	0	60 ± 8*	0	87 ± 9**

Values are given as mean ± SEM (n = 10).

[a] Given for ethanol-treated rats only: pair fed controls received the same amounts.

*: Significantly different from blood ethanol levels at 9 am, $p < 0.05$.

**: Significantly different from blood ethanol levels at 9 am, $p < 0.001$.

The daily ethanol intake per kg body weight by the BN rats was slightly higher when compared to WAG rats (Table 1). Within the BN strain the ethanol intake per kg body weight gradually increased during the treatment period. A slight gradual decrease was noted in the rats of the WAG strain.

Blood ethanol levels were slightly higher in the WAG rats when compared to BN rats. In both strains, blood ethanol levels were significantly higher at 9 p.m. than at 9 a.m. (Table 1). This may be explained by the fact that the food intake was higher in the hours after fresh diet was given (at about 10 a.m.), i.e. after the morning time point.

Light microscopy

The liver biopsies taken at 6, 9 and 12 months only showed steatosis, which was most pronounced in the ethanol-treated WAG rats. To a lesser degree, steatosis was also present in ethanol-treated BN rats as well as in both control groups. Very occasionally, minor parenchymal inflammation ('+' round cell) was noted in some biopsies of treated and control rats. No parenchymal fibrosis was found. No fibrosis was observed in central vein walls.

At sacrifice at 19 months, moderate ('+' - '++') to pronounced ('++' - '+++') steatosis of mixed micro/macrovesicular type of comparable severity was present in the ethanol-treated and in control rats of the BN strain (Table 2, Figs. 1a,b,d). The degree of inflammation was the same in both groups of control and ethanol-treated BN rats (Table 2, 1a,b) and was usually of the round cell infiltrate type. In the most severe cases, the inflammation was of the mixed round cell-polymorphonuclear cell type with spotty and occasionally focal necrosis of the liver parenchyma, and increased mixed inflammatory infiltration of portal tracts. Severe inflammation was usually associated with considerable steatosis. However, no fibrosis was present in livers of the ethanol-treated BN rats (Fig. 1c). One ethanol-treated BN rat died after 11 months due to severe ulcerative purulent oesophagitis with microorganisms, accompanied by extensive local necrosis of two liver lobes (not shown).

Table 2. Histopathological evaluation of inflammation and steatosis of liver of ethanol-treated and control rats.

Treatment strain	Inflammation				Steatosis			
	0	+	++	+++	0	+	++	+++
Control BN	0	4	6	0	0	4	5	1
Ethanol BN	0	5	5	0	0	3	6	1
Control WAG	0	9	1	0	7	3	0	0
Ethanol WAG	1	3	6	0	1	3	6	0

Values represent the number of rats within each category (n = 10).
Gradings '0' to '+++' are described in "Material and Methods".

In the ethanol-treated WAG rats, the degree and pattern of steatosis and inflammation in the liver at the time of death were comparable with those in the ethanol-treated BN rats (Table 2). In 60% of the ethanol-treated WAG rats, systemic disease was present in the cases of most pronounced parenchymal and portal liver inflammation with steatosis. However, neither parenchymal fibrosis nor thickening of the central vein walls was present. In particular, the areas of spotty or focal coagulative

Figure 1a. Liver of a BN rat, which was administered the ethanol-high vitamin A - containing diet of Lieber-DeCarli for 16 months, shows extensive (+ + / + + +), predominantly microvesicular steatosis and only minor (+ / + +) inflammation of round cell infiltrate.No fibrosis is seen. HPS 210x); inset shows some parenchymal cells with microvesicular change (500x).

Figure 1b. Serial section of a. stained for collagen. Only the portal tract (asterisk) shows normal frame work of connective tissue. Occasionally a collagen fiber is seen along the sinusoids (arrow) but no increase in collagen is evident. Sirius Red 210x.

Figure 1c. Liver of the pair fed control BN rat of fig. 1a-b. There is moderate steatosis (+ +) of mixed macro-microvesicular type, minor (+ / + +) inflammation and no fibrosis. HPS 210x.

necrosis of the severely inflamed livers were not fibrotic (Figs. 2c,d). This florid inflammation, with focal necrosis and without fibrosis, was always associated with systemic disease, usually marked interstitial pneumonia (Fig. 2a) and active chronic pyelitis (Fig. 2b). Steatosis and inflammation, and also associated systemic disease, were considerably less prominent in the control group of WAG rats than in the ethanol-treated group (Table 2). One ethanol-treated WAG rat had hepatocellular carcinoma in 2 liver lobes at autopsy (not shown). Hepatocellular carcinoma is a very rare phenomenon in our rat strains (11).

Figure 2a. Lung of a WAG rat after 16 months of ethanol-containing liquid diet, showing interstitial pneumonia with desquamation of alveolar lining cells and with presence of macrophages in the alveolar spaces. HPS 80x.

Figure 2b. Kidney of the same ethanol-treated WAG rat of a., showing medulla (star) and mucosa lining the pyelum with active inflammation (long arrow); in the lumen of the pyelum polymorphonuclear leucocytes are accumulated (short arrow). HPS 210x.

Figure 2c. Liver of the same ethanol-treated rat with active pneumonia (a) and active pyelitis (b) showing extensive (++/+++) steatosis of mixed macro- microvesicular type with a large focus of coagulative parenchymal necrosis (asterisk). HPS 80x.

Figure 2d. A liver section of the same focus of necrosis (asterisk) as shown in 2c, displaying no increase in collagen. Sirius Red 80x.

Fat-storing cells of alcohol-treated and control rats of both strains were roughly equally prominent in lipid droplets content, as judged by light microscopy. No megamitochondria were observed in hepatocytes of either ethanol-treated or control rats of both strains.

Electron microscopy and morphometric analysis

Electron microscopical analysis showed that the ultrastructural characteristics of ethanol-treated and control rat livers were qualitatively similar. In the liver lobule, fat-storing cells, containing many lipid droplets, were seen only in the Disse space. The parenchymal

Figure 3. Transmission electron microscopy of the liver of a BN rat treated with ethanol for 16 months. A fat-storing cell (F) is situated beneath the endothelial lining (arrow) in the space of Disse (sd) between the recesses of parenchymal cells (P). The cytoplasm of the fat-storing cell demonstrates lipid droplets (L), some lysosomes, and vesicular structures. Note the relative abundance of short strands of slightly dilated rough endoplasmic reticulum (RER) (arrowheads). In the space of Disse, collagen fibers were sometimes noted (not present in this illustration). Parenchymal cells display numerous microvilli (mv) at their surface. Note the normal appearance of their cytoplasm containing some short non-dilated strands of RER and lipid droplets. Original enlargement: 6800x.

cells also contained lipid droplets (of a higher electron density), but no other major ultrastructural alterations, such as megamitochondria, were evident in these cells (Fig. 3). No increase in the amount of collagen in the space of Disse was evident and no increase in the number of (myo-)fibroblasts was observed in the perivenular area, nor in the adjacent parenchyma (not shown). Quantitative morphometric analysis showed that the relative surface area occupied by fat droplets in fat-storing cells seemed to be decreased in ethanol-treated rats of both strains (Table 3), but this was only significant for the surface area of fat droplets per total cell body in BN rats. The surface area of the RER in FSC appeared increased by ethanol treatment, especially in WAG rats. No changes were found in the surface area of mitochondria in FSC. The number of FSC per 1000 hepatocytes (ITO cell index) was significantly lower in the ethanol-fed animals of both strains, particularly in WAG rats (Table 3).

Table 3. Ito cell index and morphometric analysis of the relative surface areas of RER, lipid droplets and mitochondria in FSC.

	BN		WAG	
	control	ethanol	control	ethanol
Ito cell index[a]	214 ± 16	179 ± 9*	209 ± 21	140 ± 16*
Nucleus included[b]:				
Nucleus	16.9 ± 0.5	22.5 ± 0.8*	14.8 ± 1.1	19.3 ± 3.4
Lipid	61.2 ± 1.2	53.2 ± 1.7*	67.2 ± 2.4	55.6 ± 7.1
RER	0.4 ± 0.1	0.8 ± 0.3	0.2 ± 0.1	0.8 ± 0.2*
Mitochondria	1.5 ± 0.3	1.7 ± 0.3	1.7 ± 0.3	2.1 ± 0.5
Nucleus excluded[c]:				
Lipid	73.5 ± 1.6	67.6 ± 2.2	78.9 ± 2.1	67.5 ± 6.2
RER	0.4 ± 0.1	1.1 ± 0.4	0.3 ± 0.1	1.0 ± 0.3*
Mitochondria	1.8 ± 0.3	2.3 ± 0.4	2.0 ± 0.3	2.6 ± 0.6

Values are given as means ± SEM (n = 5).
* Significantly different from control ($p < 0.05$).
[a] Expressed as number of FSC per 1000 parenchymal cells.
[b] Expressed as percentage of the total cell body (nucleus included).
[c] Expressed as percentage of the cytoplasm (nucleus excluded).
RER = Rough endoplasmic reticulum.

Table 4. Hydroxyproline content of the liver of ethanol fed and pair fed control rats.

Hydroxyproline content	BN		WAG	
	control	ethanol	control	ethanol
μg/g liver	220 ± 20	247 ± 35	196 ± 23	193 ± 17
μg/total liver	2518 ± 400	3783 ± 679	1775 ± 209	2140 ± 154

Values are given as mean ± SEM (n = 8).

Biochemical observations

Biochemical results revealed no significant increase in the hydroxyproline content of liver tissue from ethanol-treated rats (Table 4). This is in agreement with the histopathological

observations of the absence of liver fibrosis. Results of an extensive biochemical analysis, presented in the accompanying paper (6), show that the effects of ethanol treatment on vitamin A metabolism and storage and on the appearance of liver enzymes in serum are less severe than reported (5).

DISCUSSION

The present study with a liquid diet containing ethanol and a high amount of vitamin A was initiated to further analyze the mechanisms and critical early events of ethanol-induced liver fibrosis in a rat model. This ethanol-containing diet was reported to be nutritionally adequate (5,7). In 1983, Leo and Lieber (5) showed that perivenular fibrosis of the liver, with thickening of the central vein, developed within 9 months in all Spraque-Dawley rats treated with this diet supplemented with a high amount of additional vitamin A.

We have applied the same treatment protocol in two rat strains, BN and WAG, which are bred and commonly used in our institute. We did not succeed in eliciting fibrosis with the ethanol-high vitamin A-containing Lieber-De Carli diet in either strain, even after the duration of the experiment was extended to 16 months. It is unlikely that the absence of fibrosis is due to a strain specific response, since earlier studies performed in our institute have shown that both BN and WAG rats are at least as sensitive to the induction of liver fibrosis by CCl_4 (12) and heterologous serum (unpublished results) as reported for other strains, including Sprague-Dawley (13). In BN rats, it was also established that ethanol could potentiate liver fibrosis previously induced with CCl_4 (9). In a preliminary experiment with the ethanol-containing Lieber-De Carli liquid diet, which contained twice the normal amount of vitamin A and was carried out in the same two rat strains and in Sprague-Dawley rats, we found no fibrosis after 12 months and saw only minor to moderate steatosis of the liver (unpublished results).

The intake of the ethanol-high vitamin A diet per kg of body weight of both strains was similar to the intake of the Sprague-Dawley rats fed the same diet in the experiments of Leo and Lieber (5). They reported that the total dietary intake of the Sprague-Dawley rats kept on the ethanol-high vitamin A diet for 9 months was only 2/3 of that consumed by the rats on the ethanol-normal vitamin A diet for the same period. Although the data for the ethanol-high vitamin A group were not calculated by Leo and Lieber (5), it can be inferred from Lieber's 1989 update (7) that rats fed the ethanol-high vitamin A diet consumed about 8-10 g/kg body weight and reached blood levels of 66-100 mg ethanol/dl blood. This shows that the absence of hepatic fibrosis in the present study is not attributable to a lower ethanol intake or lower blood ethanol levels.

The induction of liver fibrosis by the ethanol treatment also might be dependent on

the age of the rats at the start of treatment. In our experience, administration of ethanol to weanlings affected their maturation and growth, and resulted in bad general health (data not shown). These alterations were not observed when the diet was administered to 3-month-old rats. Developing animals may be more sensitive to ethanol and vitamin A toxicity in general and this could contribute to the development of liver fibrosis.

In the present study, the only histological findings in the liver common with those reported by Lieber's group (5) were steatosis and round cell inflammatory infiltration. In the most pronounced cases, steatosis and inflammation were located in the same area's of the liver as reported by Lieber and coworkers (zone 2 and 3 according to Rappaport's classification; Figs. 1a,b). In the BN rats, however, steatosis and inflammation were roughly equally prevalent in the pair fed controls and in the ethanol-treated animals. In the WAG rats, steatosis was clearly more pronounced in the ethanol-treated group. Steatosis was not present in the control rats used by Leo and Lieber (5).

Steatosis has been interpreted as a direct effect of ethanol on the liver (14). However, a high fat content of the diet and the fatty acid composition may strongly influence the development and the degree of steatosis (15,16). Our finding of steatosis in livers of control rats supports this.

Striking features in the present study were severe focal necrosis in the steatotic liver of ethanol-treated rats. These lesions were not, however, reminiscent of human alcoholic hepatitis because of their sparsity, large size, and coagulative nature and because they lacked the highly characteristic (17) fibrosis of the lesions of the human disease. Focal necrosis was only seen in the livers of ethanol-treated rats with severe extrahepatic inflammation. Therefore, chronic ethanol administration in rats might, as in humans, have an indirect effect on the liver by making the organism more sensitive to noxious agents (infections, toxins). In this way it could have an effect on the rat liver similar to (non-specific) reactive hepatitis observed in human liver in the course of infectious diseases such as pneumonia or septicaemia.

In the reports of the experiments of Lieber and coworkers, the histopathological findings of the extrahepatic organs at sacrifice were not mentioned (5). Therefore, it is possible that the liver fibrosis in their experiments is the result of a combination of malnutrition (as argued by Rao *et al.*,18), of genetic factor(s) specific for the rat strain used by Lieber's group (Sprague-Dawley), and of superimposed systemic or infectious disease related to chronic ethanol consumption. This conclusion is in line with results of recent epidemiological studies (19). In humans, overconsumption of ethanol is often associated with malnutrition and with increased susceptibility to infectious disease due to interaction of ethanol intake with the immune system (14,19).

Quantitative morphometric analysis of the livers of ethanol-fed rats of both strains revealed dilatated RER and reduced lipid droplet content of fat-storing cells, which can be considered as very early signs of transition of fat-storing cells to fibroblast-like cells

(12,20,21). In our study, however no increase in such precursor cells of fibrosis was found, and there was no increase in collagen deposition. The observed decrease in Ito-cell index might be explained by the increased liver volume due to steatosis, rather than to an absolute decrease in the number of FSC. In ALD, another important precursor cell of liver fibrosis has been identified, called myofibroblast (4,5,21,22). These cells are a normal part of the adventitia of the central vein and are held responsible, by proliferation and migration, for the fibrosis of the wall of the central vein (perivenular fibrosis or sclerosis) and for the pericellular parenchymal fibrosis of zone 3 (22,23). In our rats, however, no such alterations of myofibroblasts were found.

Our results show that long-term treatment of rats with ethanol in the vitamin A supplemented Lieber-De Carli diet does not necessarily result in liver fibrosis. Therefore, the conclusions of Leo and Lieber (5) cannot be generalized and the role of vitamin A as a major factor in the pathogenesis of alcoholic liver fibrosis should be reconsidered. Other factors, including age, the occurrence of extrahepatic lesions and immunological, nutritional and metabolic characteristics probably play a more important role.

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B. BIOCHEMICAL OBSERVATIONS

ABSTRACT

The inability of the "ethanol/high vitamin A Lieber-DeCarli diet" to induce liver fibrosis in two different rat strains was further evaluated by determining changes in parameters of liver cell damage and of retinoid and lipid metabolism. In the ethanol/vitamin A treated group, slight but constant hepatic cell damage, as indicated by elevated alanine aminotransferase, aspartate aminotransferase and glutamate dehydrogenase activities in blood, was already observed at 6 months and maintained until the time of sacrifice at 16 months. Serum gamma glutamyl transaminase activities were not raised. Moderate parenchymal liver cell damage was not accompanied by fibrosis. Hypertriglyceridemia or hypercholesterolemia were observed at 6-16 months of chronic alcohol administration. This response was strain dependent. In ethanol-treated rats of both strains, total liver retinoids and serum retinol concentrations were not altered. Therefore, the hypothesis that interaction between alcohol and retinoids is a major factor in the pathogenesis of alcoholic liver disease, needs to be reconsidered.

INTRODUCTION

In the accompanying paper, we report that long-term treatment of rats with the ethanol/high vitamin A diet does not necessarily produce fibrosis or other severe morphological alterations of the liver (1). These results were in apparent contrast with those obtained by Leo and Lieber using the same diet in another experimental set-up (2). In this study we report our biochemical studies in the same rat model. These were focused on parameters of cell necrosis and of vitamin A and lipid metabolism. These studies were conducted to further delineate the conditions and mechanisms that determine the degree of liver damage and disease inflicted by chronic alcohol consumption, with emphasis on the contributory role of vitamin A toxicity.

MATERIALS AND METHODS

Experimental set-up

Animals, housing conditions, treatment schedules and methods for organ preparation at sacrifice are described in detail in the accompanying paper (1).

Retinoid analysis

The content of retinol and the three main retinyl esters, *i.e.* retinyl palmitate, retinyl stèarate and retinyl oleate, were determined in liver, lung and serum using high performance liquid chromatography (HPLC) (3). Serum concentration of retinol binding protein (RBP) and liver concentrations of RBP and cellular retinol binding protein (CRBP) were analyzed by specific radioimmunoassays (4,5).

Biochemical analysis of blood and serum

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyl transaminase (GGT), triglyceride (TG) and cholesterol (CHOL) were determined in heparinized blood from the tail vein using the Reflotron system (Boehringer Mannheim, F.R.G.) after 6, 9, 12 and 16 months of liquid diet administration.

At the time of death, blood was collected from the dorsal vein. Serum glutamate dehydrogenase (GDH) activity was assayed by the method of Ellis and Goldberg (6). Lactate dehydrogenase (LDH), triglyceride (TG), cholesterol (CHOL) and phospholipids (PL) were determined with commercial test combinations (Boehringer Mannheim, FRG). Serum lipoproteins were fractionated by density gradient centrifugation ($1.006 < d < 1.210$) according to the method of Redgrave (7). Briefly, 0.65 g KBr was added to 2 ml serum resulting in a density of 1.21 and an additional 1.8 ml KBr/NaCl solution with the same density was added. Three 2.7 ml layers of KBr/NaCl solution with densities of 1.050, 1.019 and 1.006 respectively, were stacked on the serum fraction. After centrifugation (200,000 x g, 22 h, 18°C), fractions of 0.6 ml were collected. For each fraction, protein (8), cholesterol, triglyceride and phospholipid (PL) content were determined. The apolipoproteins of each fraction were analyzed by polyacrylamide gel electrophoresis according to the method of Laemmli (9).

Statistics

Results are expressed as the mean ± S.E.M. Statistical analysis was performed by the Student t-test for paired observations (significance level 5%, 1% and 0.1%).

RESULTS

Morphological alterations in liver and other tissues

A detailed description of the morphology is given in a separate paper (1). None of the rats fed the ethanol-containing diet showed liver fibrosis and the total hydroxyproline content of the livers of both strains were not significantly elevated. Fatty liver and reactive changes in liver parenchyma, with foci of congulative parenchymal necrosis and mixed cellular inflammatory infiltration, were observed and were explained as an effect

of extrahepatic diseases, *i.e.* interstitial pneumonia and pyelitis.

Retinoid content of tissue and serum

Retinoid contents (retinol and retinyl esters) of liver, lung and serum are shown in Table 1. In the WAG rats, retinol and retinyl ester concentrations per gram liver were significantly lower in the ethanol-treated animals as compared to controls; however, due to a higher liver weight, the total retinoid liver content did not change. In the BN rats, neither the concentration nor the content of retinoids changed. The total amount of retinoids stored in the liver at the time of death represented approximately 10-16% of the total intake of retinoid in the diet. In both strains, the retinyl ester composition of the liver was altered by ethanol administration. An increased oleate and stearate ester content and a decreased palmitate ester content were noted. No changes were observed in the retinol and CRBP concentrations.

Although the retinoid concentration of the lungs of BN rats fed the ethanol-containing diet was significantly higher than of the controls (Table 1), the retinyl ester composition was identical. In contrast, the retinoid concentration of the lungs of ethanol-fed WAG rats was not significantly different from their pair-fed controls, but the retinyl ester composition was changed. The percentage palmitate esters was higher and the percentage stearate esters was lower. Retinol was not detectable in lungs of WAG rats, but in BN rats of both groups 6-8% of the retinoids consisted of retinol.

Serum retinoid levels were not affected by the ethanol consumption (Table 1) and no change in retinoid composition was found; retinol was the main retinoid present in serum (95-100%). The ratio between the concentration of retinol and retinol-binding protein in serum was also not affected by ethanol treatment, which indicates that no alterations in the degree of protein binding of retinol had occurred.

Biochemical analysis of blood and serum

ALAT, ASAT, GGT and GDH activities were determined in serum as indicators of parenchymal liver cell damage (Table 2). ALAT activity was slightly increased in both the BN and WAG rats which were fed the ethanol-containing diet for 6 months. The increased activity was maintained until the animals were killed. ASAT activity was also slightly increased in the rats fed the ethanol-containing diet for 9 and 12 months, but at the time of death this activity was not longer significantly different from controls. The activity of GGT, a liver enzyme that frequently is elevated in sera of alcoholics, was below detection limit (< 5.00 IU/l, 37°C) at all time points in all rats. The liver mitochondrial enzyme GDH was slightly elevated in serum of ethanol-fed rats of both strains. These biochemical data indicate that a small but consistent parenchymal liver cell damage was induced in the ethanol-fed rats of the two strains studied.

Table 1. Effect of ethanol administration on body weight, liver weight and on retinoid levels and retinyl-ester composition in liver, lung and serum of the BN and WAG strain at the time of sacrifice.

	BN		WAG	
	control	ethanol	control	ethanol
Body weight (g)	434 ± 11	423 ± 19	428 ± 7	426 ± 11
Liver weight (g)	11.6 ± 0.7	14.0 ± 0.7*	8.1 ± 0.3	11.5 ± 1.5*
% (liver weight/body weight)	2.67 ± 0.09	3.51 ± 0.14**	1.90 ± 0.08	2.61 ± 0.29*
Retinoid and cellular retinol binding protein (CRBP) levels and retinyl ester composition (% of total retinoids) in liver				
retinol equivalent (μg/g liver)	2687 ± 430	2988 ± 250	5423 ± 290	3134 ± 312**
retinol equivalent (μg/liver)	30620 ± 6940	41902 ± 4440	44254 ± 3014	37753 ± 3731
% (liver ret. eq./consumed ret.eq.)	10.1 ± 2.3	13.8 ± 1.5	15.6 ± 1.1	13.3 ± 1.3
CRBP (μg/g liver)	24.1 ± 0.5	33.2 ± 5.4	47.8 ± 7.6	33.1 ± 3.2
% retinol	1.1 ± 0.8	0.8 ± 0.3	1.1 ± 0.8	1.2 ± 0.9
% r. oleate	3.9 ± 0.6	7.9 ± 0.3***	3.6 ± 0.7	6.9 ± 0.9***
% r. palmitate	81.4 ± 10.8	74.6 ± 0.6***	81.5 ± 10.8	75.8 ± 10.6**
% r. stearate	13.6 ± 1.4	16.8 ± 0.6***	13.7 ± 1.7	16.1 ± 1.9*
Retinoid levels and retinoid ester composition (% of total retinoids) in lung				
retinol equivalent (μg/g lung)	13.6 ± 3.0	22.2 ± 2.2**	20.4 ± 3.8	24.2 ± 2.8
% retinol	8.9 ± 4.0	6.4 ± 3.8	0.0	0.0
% r. oleate	5.9 ± 2.4	5.4 ± 1.3	3.3 ± 1.3	5.0 ± 0.7
% r. palmitate	60.2 ± 3.3	65.6 ± 3.0	63.7 ± 1.3	66.8 ± 1.4*
% r. stearate	25.0 ± 2.6	22.6 ± 1.0	33.0 ± 0.6	26.7 ± 0.8***
Retinoid and retinol binding protein levels (RBP) in serum				
retinol equivalent (μg/dl serum)	24.2 ± 3.0	22.9 ± 5.3	9.9 ± 2.7	11.1 ± 1.7
RBP (mg/dl serum)	1.84 ± 0.1	1.86 ± 0.2	1.12 ± 0.1	1.25 ± 0.2
sRBP serum/retinol (mmol/mmol)	1.15 ± 0.2	1.51 ± 0.3	2.33 ± 0.6	1.59 ± 0.2

Values are given as mean ± SEM.

* significantly different from control, $p < 0.05$

** significantly different from control, $p < 0.01$

*** significantly different from control, $p < 0.001$

Table 2. Values of some blood[1] and serum parameters of liver damage (ALAT, ASAT, GDH and LDH) and lipid metabolism (TG, CHOL and PL) for rats of both strains fed the control or ethanol diet for 6, 9, 12 or 16 months.

	BN		WAG	
	control	ethanol	control	ethanol
ALAT (U/l)[1]				
6	29.5 ± 0.6	50.2 ± 3.7**	28.2 ± 3.1	62.4 ± 20.7**
9	39.0 ± 3.0	54.2 ± 3.7**	47.2 ± 2.8	65.4 ± 2.8**
12	33.7 ± 1.5	53.0 ± 3.9*	46.0 ± 2.9	58.3 ± 1.7*
16	34.3 ± 2.1	59.9 ± 9.1*	33.4 ± 2.1	51.7 ± 2.2*
ASAT (U/l)[1]				
6	70.4 ± 20.5	108.7 ± 35.7	61.0 ± 20.7	86.5 ± 6.4
9	88.7 ± 6.1	112.5 ± 5.7*	72.5 ± 5.6	98.2 ± 3.6**
12	70.4 ± 2.3	113.4 ± 9.0*	77.0 ± 7.0	82.3 ± 3.2
16	88.8 ± 5.4	124.8 ± 17.2	72.4 ± 2.8	81.7 ± 3.2
GDH (U/l)				
16	2.2 ± 0.4	19.5 ± 3.2***	2.3 ± 1.0	16.8 ± 6.5***
LDH (U/l)				
16	1625 ± 461	1446 ± 444	1435 ± 278	1409 ± 388
TG (mmol/l)				
16	1.55 ± 0.21	2.51 ± 0.45*	1.55 ± 0.24	1.72 ± 0.20
CHOL (mmol/l)				
16	2.24 ± 0.1	2.33 ± 0.1	2.03 ± 0.10	2.98 ± 0.23***
PL (mmol/l)				
16	1.72 ± 0.10	1.97 ± 0.10	1.54 ± 0.07	2.07 ± 0.15*

Values are given as mean ± SEM.
* significantly different from control, $p < 0.05$
** significantly different from control, $p < 0.01$
*** significantly different from control, $p < 0.001$
ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; GDH = glutamate dehydrogenase; LDH = lactate dehydrogenase; TG = triglyceride; CHOL = cholesterol; PL = phospholipid.

The influence of ethanol intake on lipid metabolism, as reflected by serum triglyceride, cholesterol and phospholipid concentrations, was strain dependent (Table 2). TG, but not CHOL and PL were increased in the BN rats fed the ethanol-containing diet for 16 months. In contrast, the serum TG concentrations did not change in the

ethanol-fed WAG rats, but CHOL and PL levels were significantly increased at the time of death. Analysis at intermediate time points showed that the ethanol-induced elevations of lipids described above were already present after 9 and 12 months of treatment (data not shown). Fractionation of the serum lipoproteins of the ethanol-fed WAG rats revealed increased cholesterol levels in fractions with a density corresponding to that of HDL (Fig. 1). The phospholipid and triglyceride content were not significantly altered in either the HDL, LDL or VLDL rich fractions. No alterations were observed in the fractions isolated from the ethanol-fed BN rats (data not shown). Analysis by SDS-polyacrylamide gel electrophoresis of the apolipoprotein profiles revealed no qualitative differences due to ethanol intake (data not shown) in either strain.

Figure 1. Density fractionation of the serum lipoproteins of the control- and ethanol-fed WAG/Rij rats. Cholesterol content (CHOL) of the ethanol-fed rats was significantly increased in the fractions with a denstity of approximately 1.142, corresponding to the HDL-rich fraction of the WAG rats. The phospholipid (PHOS) and triglyceride (TRI) contents were not significantly altered. Values are given as mean ± SEM. *Significantly different from control, $p < 0.05$. (a) cholesterol content; (b) phospholipid content; (c) triglyceride content.

DISCUSSION

The morphological data reported in the accompanying paper (1), showed that the ethanol-induced abnormalities in liver were much less severe in our rats as compared to rats fed the same diet by Leo and Lieber (2). This biochemical study was undertaken to investigate alterations in key parameters for the effects of alcohol on liver function. The results are discussed in relation to the apparent discrepancy between the two studies, and to further clarify the mechanisms responsible for the pathogenesis of alcoholic liver fibrosis.

Liver cell damage

The slightly elevated ALAT, ASAT and GDH activities and the low GGT activities in blood of the ethanol-high vitamin A fed rats of both strains indicated that slight but constant parenchymal liver cell damage was present during the major part of the experimental period. This corresponds well with the histology of the liver biopsies, which showed minimal non-specific reactive inflammation and local parenchymal cell loss (1). Leo and Lieber found that ALAT, ASAT and GDH activities of Sprague-Dawley rats were markedly elevated after 9 months treatment with the ethanol/high vitamin A diet (2). Since parenchymal cell damage is a recognized factor in liver fibrogenesis (10,11), the relatively low degree of liver damage could partly explain the absence of fibrosis in the livers of the rat strains used in our experiments.

Retinoids and their metabolism

The liver retinoid contents of the two rat strains fed high vitamin A diet for 16 months were 3 times higher than those in rats of the same age fed *ad libitum* with normal lab chow (12). The retinoid content was comparable to the livers of Sprague Dawley rats administered the diet for 9 months (2). This indicated that there are no strain related differences in the normal intestinal absorption and hepatic storage of dietary vitamin A among the various strains.

Both short and long-term administration of ethanol can seriously affect retinoid metabolism (13-16). The ethanol-induced reduction of up to 30% of hepatic stores, which has been described by several groups (13-16), was absent or insignificant in our study. In accord with others, there was less palmitate and more oleate and stearate among retinyl esters in liver, but the shift was relatively small.

Plasma levels of retinol and retinol binding protein RBP were not altered by chronic ethanol intake in rats of either strain. This indicates that, a) the regulation of the secretion of retinol-RBP complex by the liver was not affected, b) there is no risk of toxicity due to free retinol in plasma, and, c) availability of retinol for peripheral tissues was not affected in the ethanol-fed rats. Retinoid content in the lungs was somewhat

elevated in BN rats treated with ethanol and the retinyl ester composition of lung was altered in WAG rats. These changes appear to be due to a local effect of ethanol on retinoid metabolism in the lung, rather than to an increased or altered transport of retinol from liver to lung, as described by Leo and Lieber (2).

From these results it is obvious that the effects of long-term ethanol feeding on retinoid metabolism in rats are dependent on the genetic background. Some ethanol-induced alterations of retinoid metabolism were observed, but were not significant. Fatty liver, which is the result of increased retention of dietary fatty acids in the liver (17), was mildly induced by chronic ethanol consumption in our rats. Since oleate constituted about 55% of the dietary fat, the change in the acyl pattern of hepatic retinyl esters may be due to a relative increase in oleate over palmitate as substrate for lecithin:retinol acyltransferase (18).

Our results show that high vitamin A intake per se does not necessarily form a toxic co-factor for the hepatotoxicity of ethanol. If vitamin A acts as co-factor, as described by Leo and Lieber, this is probably restricted to conditions in which there is a clear disturbance of retinoid metabolism, notably a decrease in retinyl ester content in the liver. The key event may be the catabolic oxidation of retinoids into toxic metabolites by ethanol-inducible microsomal oxidases, such as the microsomal ethanol-oxidising system (MEOS) (19). In our rats, catabolism of retinoids was probably not increased by ethanol, since liver stores were not affected.

The results confirm and extend earlier observations on the dual role of vitamin A in chemically induced liver damage and fibrogenesis (20,21). In BN rats vitamin A combined with CCl_4 displayed acute toxicity, but under the right conditions vitamin A could significantly reduce liver fibrosis (20). Vitamin A appears to interact with fibrogenesis in various, antagonistic ways; by metabolic interaction with hepatotoxins resulting in cell damage, and by modulating inflammatory processes and tissue repair. Alterations in function, differentiation and proliferation of both Kupffer and fat-storing cells are likely to be involved.

The influence of ethanol on serum lipids and lipoproteins

Depending on the rat strain, our study showed that long-term ethanol consumption induced hypertriglyceridemia or hypercholesterolemia. These results reflect those obtained from clinical and epidemiologic studies in alcoholic patients, in which hypertriglyceridemia and/or hypercholesterolemia were commonly observed (22-24). The mechanism behind these effects of ethanol is not clear. Hypertriglyceridemia has been suggested to be the result of changed lipoprotein metabolism in the alcoholic liver (25,26). Ethanol administration in baboons enhanced hepatic production of VLDL triglicerides (27). In the ethanol-fed rats of the BN strain, hypertriglyceridemia was associated with slight liver cell injury, but there were no indications of enhanced, specific

production of VLDL triglycerides in these rats as indicated by the lipoprotein fractions. No significant elevations were observed in either the VLDL, LDL or HDL fractions.

This study also shows that ethanol-induced liver cell damage in the rat does not necessarily lead to hypertriglyceridemia. Hypercholesterolemia was observed in the ethanol-fed rats of the WAG strain without a rise of triglyceride levels. Fractionation of the serum lipoproteins revealed increased cholesterol levels of the HDL fractions. *In vivo* (28) and *in vitro* (29) studies indicate that HDL promotes cholesterol efflux from cells, which has been associated with a lower incidence of coronary heart disease (30), suggesting that ethanol consumption may exert protective cardiovascular effects.

The rat shows different aspects of the interaction between ethanol and lipid metabolism, and the ultimate effect was dependent on the genetic background of the strain in use. In view of the close resemblance to a variety of effects observed in humans, the use of different rat strains may contribute to the explanation of the interaction of ethanol, lipid metabolism and genetic background.

Concluding remarks

These experiments show that long-term treatment of BN and WAG rats with ethanol in the retinoid supplemented Lieber-DeCarli diet does not result in liver abnormalities that are characteristic for human ALD. The slight inflammatory response of the liver parenchyma was non-specific and had no characteristics of alcoholic hepatitis as observed in humans (1). The results also cast doubt on the hepatotoxic potential of retinoids in alcoholic liver disease (2). The role of retinoids in the pathogenesis of liver fibrosis should be reconsidered. As has been observed in experiments with other fibrotic agents like CCl_4 and heterologous serum (20,21), it is possible that high levels of retinoids in the livers of the ethanol-treated rats may suppress the induction of liver fibrosis. This may be due to influences of retinoids on the metabolism of fat-storing cells (20). Orally administered retinoids are normally transported to and stored in fat-storing cells (12,31,32). Several studies have shown that fat-storing cells are involved in collagen synthesis during fibrogenesis. Furthermore, the fibroblast-like cells associated with the fibrotic septa may be derived from fat-storing cells by migration, transformation and cell division (33-35). High retinoid concentrations, stored in the fat-storing cells, may suppress the collagen synthesizing capacity of these cells and prevent their transformation to fibroblast-like cells as has been seen in cultured fat-storing cells which were loaded with retinoids before isolation (36). In ethanol-fed rats of both strains, neither proliferation of fat-storing cells nor the appearance of fibroblast-like cells were observed, which may be due to the maintenance of high retinoid content in the fat-storing cells.

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CHAPTER 3

ALCOHOL IN COMBINATION WITH A PROTEIN-DEFICIENT HIGH FAT DIET CAUSES INCREASED LIVER FIBROSIS IN RATS

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SUMMARY

Long-term severely-malnourished rats on a fat-rich, calorie-sufficient but otherwise poly-deficient liquid diet, composed of mashed potatoes with mayonnaise, showed increased liver fibrosis and cirrhosis when alcohol, as whisky, was incorporated into the diet.

Malnutrition may be an important factor in the development of the fibrosis of alcoholic liver disease.

INTRODUCTION

Until three decades ago, it was generally thought that the malnutrition found in alcoholics was a cause, not a consequence of alcoholic liver disease (1,2). Since then, evidence has grown that the nutritional deficiency associated with chronic alcohol abuse should be considered a secondary phenomenon. Nutritional deficiency is caused by intestinal, pancreatic and liver dysfunction due to the harmful effects of alcohol on the gut, pancreas and liver which lead to nutrient malabsorption from the gut. The increasing awareness that malnutrition is a secondary phenomenon of chronic alcohol abuse has been supported by results gained from experimental models for alcohol-induced liver damage in rats and baboons as developed by Lieber and co-workers. The so-called Lieber-DeCarli diet for rats is a liquid diet composed to be nutritionally adequate into which is incorporated a large quantity of ethanol (3). The results gained from baboons indicated a direct hepatotoxicity of alcohol and seemed to exclude the possibility that nutritional factors contributed to the liver damage incurred, namely steatosis, alcoholic hepatitis followed by fibrosis and cirrhosis (4). The Lieber-DeCarli diet administered to rats did not result in liver damage beyond the stage of steatosis unless it was supplemented with a large amount of vitamin A. In that case, steatosis and extensive fibrosis in the livers of all rats were observed after 9 months of diet intake but alcoholic hepatitis was not found (5). However, the induced liver fibrosis as demonstrated by Lieber's group with alcohol administration to experimental animals has not been confirmed by others, neither in baboons (6), Rhesus monkeys (7,8) nor in rats (9). Moreover, the nutritional adequacy of the Lieber-DeCarli formula is questionable (1,10).

We have previously reported on the enhancing effect of ethanol on liver fibrosis induced in rats which had been pretreated by carbon tetrachloride (11). We can now report that in severely-malnourished rats fed a fat-rich but otherwise broadly-deficient diet, liver fibrosis and cirrhosis were considerably enhanced by administration of alcohol given as whisky.

MATERIAL AND METHODS

Animals and diet

Male WAG/Rij rats (n = 16), aged three months, weighing 271 ± 5 g, were obtained from the Institutes colony (Rijswijk, The Netherlands). These specific pathogen-free derived rats were maintained under "clean conventional conditions" (12). The diet was a liquid alcohol-containing diet composed of powdered mashed potatoes (manufactured by Nestlé Nederland B.V., Amsterdam, the Netherlands; 101 g/l) and mayonnaise (Calvé, Delft, the Netherlands; 75 g/l) as the only source of food and fluid. It contained 37% of the total calories either as ethanol, namely whisky (MacTrevor, Scotch whisky), or isocaloric carbohydrates (sucrose), 3% as protein, 38% as fat, and 22% as additional complex carbohydrate. 1 litre of diet contained 120 mg of choline chloride, 50 IU of vitamin A, 1.40 mg of zinc and 330 μg of copper (TNO Nutrition, Zeist, the Netherlands). The liquid diets were freshly prepared daily in the laboratory using a kitchen-type blender. All ingredients were homogenised with water at 60°C for three minutes. The diet permitted pair feeding of alcohol-treated with non-alcohol treated matched control rats. Body weight was measured every week. The rats were sacrificed after 12 months of liquid diet administration at the age of 15 months.

Biochemical analysis

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyl transaminase (GGT), triglyceride (TG) and cholesterol (CHOL) were determined in heparinized blood from the tail vein using the Reflotron system (Boehringer Mannheim, Mannheim, Germany) after 3, 6 and 12 months of liquid diet administration. Blood ethanol levels were determined with a commercially-available test combination (Boehringer Mannheim, Mannheim, Germany) on heparinized blood collected from the tail vein in the morning (9 a.m.) and in the evening (9 p.m.).

Surgical liver biopsies

Surgical liver biopsies were taken at 3, 6 and 9 months of liquid diet administration to assess liver morphology in the course of the experiment. The rats were anaesthesized by halothane and wedge shaped sections of 20 to 30 mg of the median lobe were removed with an iris scissor (Stöpler, Utrecht, The Netherlands). The liver biopsy was fixed in 4% buffered formalin for light microscopy. The bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, the Netherlands) and the peritoneum and the abdominal wall were sutured with absorbable surgical material (4-0 Dexon plus, Davis + Geck, Manati, USA). The skin was closed with wound clips (Clay Adams, Parsippany, USA) which were removed one week after surgery.

Organ preparation and histopathology

After 12 months, it was decided that the experiment should be terminated because of the very poor general health of the animals especially of the alcohol-treated rats (Table 1). Animals were fasted overnight before being killed. At the time of death, the livers were perfused with phosphate-buffered saline (PBS, pH 7.4) through the portal vein for 5 min at 5 ml/min, followed by perfusion fixation. The median lobe was excluded from fixation by ligation and, after removal from the body, was weighed and immediately frozen in liquid nitrogen and stored at -70°C for later biochemical analysis. The left lateral lobe was temporarily excluded from perfusion by application of a loose ligation. The right lateral and the caudate liver lobe were then fixed by perfusion with 2.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 5 ml/min for 8 min) for electron microscopy. The ligation of the left lateral lobe was removed and this lobe was then fixed by perfusion with 4% buffered formalin for light microscopy. After fixation, the liver was removed from the body and weighed. A routine post mortem examination was carried out and kidney, left lung, heart, spleen and brain were fixed in neutral buffered formalin for light microscopy. The tissues were routinely processed after fixation by embedding in paraplast and sectioning at 4 micron thickness. Sections of all tissues were stained with haematoxylin-phloxin-saffron (HPS). Liver sections were additionally stained with periodic acid Schiff (PAS) with and without diastase pretreatment, elastica van Gieson and Sirius-Red-F3BA (13) for connective tissue and collagen, respectively.

Light microscopy of the liver

Morphology was assessed by a pathologist who worked blind as to the source of the specimens. Liver sections were analysed for the presence of parenchymal and portal damage, notably steatosis, inflammation, fibrosis and cirrhosis.

Steatosis was graded from 0 to +++, reflecting no steatosis, an estimated 1/3, 2/3 and all parenchymal cell involvement, respectively. Predominance of the steatosis as micro- or macrovesicular change was noted. Inflammation was graded from 0 to ++++. Grading + reflected the presence of scattered isolated inflammatory cells in the parenchyma, ++ focal parenchymal collection of inflammatory cells, +++ focal collection in parenchyma and in fibrous septa of inflammatory cells, and ++++ diffuse inflammation. Predominance of mononuclear or polymorphonuclear inflammatory cell response was noted.

Fibrosis was graded from 0 to ++++. Grade + reflected focal fibrosis of the parenchyma and/or portal tracts, ++ the presence in the parenchyma of incomplete fibrous septa, +++ complete dissecting fibrous septa and ++++ the presence of cirrhosis with regeneration nodules. Intermediate grading of steatosis, inflammation and fibrosis was applied when necessary.

Table 1. Body weight, dietary and ethanol intake, blood ethanol levels, liver weight and ratio liver/body weight of WAG/Rij rats kept on liquid diet with or without ethanol for 0, 3, 6, 9 and 12 months.

	control	ethanol
Body weight (g)		
0 months treatment	270 ± 4	272 ± 5
3 months	238 ± 2	233 ± 4
6 months	215 ± 4	197 ± 8*
9 months	208 ± 5	189 ± 9*
12 months	201 ± 5	161 ± 10**
Dietary intake (ml/kg/day)[a]		
3 months	-	132 ± 4
6 months	-	185 ± 6
9 months	-	186 ± 6
12 months	-	200 ± 11
Ethanol intake (g/kg/day)		
3 months	0	7.8 ± 0.2
6 months	0	10.9 ± 0.3
9 months	0	11.0 ± 0.4
12 months	0	11.9 ± 0.6
Blood ethanol levels (mg/dl)		
11 months (9 am)	0	55 ± 7
11 months (9 pm)	0	114 ± 10[b]**
Liver weight (g)		
12 months	11.6 ± 0.7	9.8 ± 0.9*
% (liver/body weight)		
12 months	5.7 ± 0.3	6.0 ± 0.3

Values are given as mean ± SEM (n = 8).
[a]: Given for ethanol-treated rats only: pair fed controls received the same amounts.
*: Significantly different from control, $p < 0.05$.
**: Significantly different from control, $p < 0.01$.
[b]**: Significantly different from blood ethanol levels at 9 am, $p < 0.01$.

Electron microscopy

For electron microscopy, random samples were taken from the right lateral and the caudate liver lobe. The samples were postfixed in 1% osmium tetraoxide, dehydrated in graded concentrations of ethanol and embedded in epon LX 112 (Ladd Research Industry Inc., Burlington, VT, USA). Ultrathin fibratome sections were mounted on

uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined with an EM 410 electron microscope (Philips, Eindhoven, the Netherlands). Fat-storing cells, Kupffer cells and pit cells were counted and expressed as the number per 100 endothelial cells.

Retinoid analysis

The content of retinol and the three main retinyl esters, viz. retinyl palmitate, retinyl stearate and retinyl oleate, were determined in liver and plasma using high performance liquid chromatography (HPLC) (14).

Hydroxyproline determination of liver tissue

Three to five hundred mg of the frozen median liver lobe were processed for hydroxyproline determination as recently described (9).

Statistics

Results are expressed as the mean ± SEM. Statistical analysis was performed by the Student's t-test for paired observations (significance level 5%, 1% and 0.1%).

RESULTS

Dietary and alcohol intake, and blood ethanol levels

Dietary and alcohol intake, body weight, blood ethanol levels, liver weight and ratio liver/body weight are shown in Table 1.

The daily intake of liquid diet per kg body weight gradually increased in the course of the experiment. The body weight curves during the treatment period were different for ethanol-treated and control rats. A gradual decrease in body weight was noted in all rats but the ethanol-treated rats lost significantly more weight than their matched controls. The daily ethanol intake per kg body weight gradually increased during the treatment period. Blood ethanol levels were significantly higher at 9 p.m. than at 9 a.m. (Table 1). This may be explained by the fact that the food intake was higher in the hours after fresh diet was given at about 10 a.m.

Light microscopy

Three months into the experimental treatment, the liver biopsies of both control and alcohol-treated rats showed microvesicular steatosis in acinar zones II and III, which was most pronounced in the ethanol-treated rats (not shown). Macrovesicular steatosis was present in acinar zones II and III of alcohol-treated rats only. At 6 months, micro- and macrovesecular steatosis was present throughout the parenchyma of both control and

alcohol-treated rats. Very occasionally, bile duct cell proliferation was noted in biopsies of alcohol-treated and control rats. Nine months into the experimental treatment, focal parenchymal fibrosis was found (+) in 3 out of 8 biopsies of alcohol-treated rats. Focal fibrosis was associated with minor parenchymal inflammation (+).

The findings in the livers at termination of the experiment after 12 months are summarized in Fig. 1. Steatosis was severe in all livers of both alcohol-treated and control rats (Fig. 2). It was predominantly of the macrovesicular type and it was most pronounced in acinar zone II. Inflammation was only present accompanying fibrous septa. Inflammatory cells were mixed polymorphonuclear and mononuclear cells. Fibrous septa found in 2 of 8 control rats were incomplete (Fig. 2a,c). Complete fibrous septa were found in all 8 alcohol-treated rats of which 4 showed transition to cirrhosis by formation of regeneration nodules (Fig. 2b,d). Fibrosis was predominant in acinar zone III but centro-portal fibrous bridges with proliferation of bile ductules were always found in fibrosis grade +++. Thickening of the wall of the hepatic (central) vein was not observed. A striking finding was the irregular distribution of the fibrosis. Often, one part of the liver lobule displayed only steatosis, while various degrees of fibrosis and even cirrhosis were found in the remaining part.

Figure 1. Histopathological evaluation of abnormalities in liver of control and ethanol-treated rats at sacrifice. Histopathological scoring of steatosis, inflammation and fibrosis was done as described in material and methods. **: Values (mean ± SEM) are significantly different from those of the control group ($p < 0.01$). ***: Values (mean ± SEM) are significantly different from those of the control group ($p < 0.001$).

Ultrastructural observations

Electronmicroscopical examinations showed fat depositions in parenchymal cells both in rats fed the control diet and in those fed the alcohol diet for 12 months (Fig. 3a-d). No other major ultrastructural alterations, such as megamitochondria, were evident in parenchymal cells of either group. Many bile duct cells were situated in an unusual location near the space of Disse between parenchymal cells in livers of both groups (Fig 3a,b).

Figure 2a-d. Micrographs of a liver of a control and a cirrhotic ethanol-treated rat.
In the control (a), collagen is present as normal supportive connective tissue in portal tracts (arrow) and in the wall of central veins (arrowhead), whereas in the ethanol-treated rat (c) abundant collagen is present around the parenchymal nodules throughout the liver. A higher magnification of (a) and (c) are shown in (b) and (d). Apart from collagen in portal tracts and in the walls of the central veins, only tiny collagen deposits (arrows) are focally present in steatotic, but otherwise normal parenchyma (b). In the liver of the ethanol-treated rat, the steatotic irregularly shaped parenchymal nodules are surrounded by a variable amount of collagen containing portal vessels and bile ducts (d).
Sirius-Red staining; a, c (x 4); b, d (x 40).

In the control livers, fat-storing cells, with few lipid droplets, were seen only in the Disse space, and they sometimes revealed an activated appearance as assessed by the presence of some short dilated strands of RER (Fig. 3c). In alcohol-treated rats, activation of fat-storing cells was more pronounced, as judged from the dilated RER and increase in lysosomes (Fig. 3d). Lipid droplets were not often seen.

Collagen was prominently present in the space of Disse of both alcohol-treated rats and pair-fed controls (Fig. 3c,d). However, the increase in collagen and in the number of myofibroblasts or fibroblasts observed in the perivenular area, and in the adjacent parenchyma was only seen in the alcohol-treated rats. Ultrastructural observations of the septa revealed fibroblasts, bile duct cells and Kupffer cells surrounded by collagen fibrils and fuzzy material (Fig. 3e). Dilated strands of RER were present in these fibroblasts. Pit cells, characterized by an oval nucleus, pale cytoplasm containing dense granules and well-developed pseudopodia, were regularly seen in the perisinusoidal space next to fat-storing cells of livers of control rats (Fig. 3c), but not in those of alcohol-treated rats.

Figure 3a-e. Transmission electron micrographs of livers of control and ethanol-treated rats at sacrifice.

a. Low power magnification of a perisinusoidal area of the liver of a control rat showing a duct of Hering (H) near the space of Disse. Parenchymal cells (P) contain abundant lipid droplets (L). S: sinusoid; B: bile duct cell (2.667 x).

b. Electron microscopical overview of a liver of an ethanol-treated rat with a bile duct cell (B) surrounded by a basal lamina (not visible at this magnification) near the space of Disse. A fat-storing cell (F) with some lipid droplets (L) and extended RER (arrow) is present in the space of Disse (arrowhead). Parenchymal cells (P) contain numerous large lipid droplets (L). E: endothelial cell; S: sinusoid (2.667x).

c. Control rat. A fat-storing cell (F) with swollen RER is lying in the space of Disse next to a pit cell (P). The inset shows a pit cell in mitosis. S: sinusoid; C: collagen (4.417 x; inset 3.667 x).

d. Ethanol-treated rat. A fat-storing cell (F) is associated with a Kupffer cell (K) in the perisinusoidal space. Note the collagen depositions (C) around the fat-storing cell. S: sinusoid (3.583 x).

e. Ethanol-treated rat. Overview of a fibrotic septum containing fibroblast-like cells (F), Kupffer cell (K), bile duct cells (B), which are surrounded by collagen (C) and fuzzy material (arrow). (2.667 x).

The number of fat-storing cells per 100 endothelial cells in the ethanol-fed animals did not differ from that in age-matched rats of the same strain maintained on normal lab chow, but it was approximately 1.5 times the number found in control rats (Table 2). The control livers contained an 80% lower number of Kupffer cells (expressed per 100 endothelial cells) when compared with livers of rats of the same strain on normal lab chow (Table 2). The number of Kupffer cells per 100 endothelial cells in the ethanol-fed rats was approximately 5 times the number found in pair-fed controls (Table 2). The control livers contained approximately 25 times more pit cells than livers of alcohol-

treated rats (Table 2). Proliferation of pit cells was observed as indicated by the presence of mitotic figures (inset of Fig. 3c).

Table 2. Number of fat-storing, Kupffer cells and pit cells per 100 endothelial cells in liver of healthy rats of the WAG/Rij strain (ref.15) and in livers of rats fed the high fat - low protein control and ethanol-containing diet.

	healthy (ref.15)	control	ethanol
fat-storing cells	85 ± 1	53 ± 11*	75 ± 4
Kupffer cells	49 ± 10	9 ± 2**	48 ± 11
Pit cells	nd	17 ± 6*	0.7 ± 0.3

Values are given as mean ± SEM (n = 3).
*: Significantly different from healthy and ethanol, Student t-test $p < 0.05$.
**: Significantly different from healthy and ethanol, Student t-test $p < 0.01$.

Biochemical observations

ALAT, ASAT and GGT activities were determined in blood as indicators of parenchymal liver cell damage (Table 3). ALAT and ASAT activities were higher in the ethanol-fed rats at three months than in the controls (normal levels) and increased activity was maintained until the animals were sacrificed. The activity of GGT, a liver enzyme that is frequently elevated in sera of alcoholics (16), was below detection limit until 12 months in all rats, but the activity was significantly increased in the ethanol-treated rats at the time of sacrifice (Table 2). These biochemical data indicate that parenchymal liver cell damage was induced in the ethanol-fed rats.

The influence of ethanol intake on lipid metabolism, as reflected by blood triglyceride (TG) and cholesterol (CHOL) concentrations, is shown in Table 3. Elevations of CHOL levels were already evident after 6 months of alcohol treatment, and both TG and CHOL were significantly increased in the rats fed the ethanol-containing diet for 12 months.

Total hepatic retinoid content (retinol plus retinyl esters) and the retinol and retinyl ester composition of the liver at sacrifice are shown in Table 4. Both in control and alcohol-fed rats, retinol and retinyl ester concentrations per gram liver were very low when compared with rats of the same age maintained on normal lab chow (17). In rats of both groups, the retinol and retinyl ester composition of the liver was altered as a result of the diet. When compared with normal healthy rats of the same age (17), less retinol and more of its esters was found.

Table 3. Values of some blood parameters of liver damage (ALAT, ASAT and GGT) and lipid metabolism (TG and CHOL) for WAG/Rij rats fed the control or ethanol diet for 0, 3, 6 or 12 months.

	control	ethanol
ALAT (U/l)		
0	45.2 ± 2.0	46.5 ± 1.3
3	19.9 ± 1.0	67.2 ± 9.1**
6	22.4 ± 2.4	48.6 ± 4.7**
12	21.2 ± 1.1	56.7 ± 9.2*
ASAT (U/l)[1]		
0	70.3 ± 4.1	63.5 ± 4.4
3	69.9 ± 9.0	174.8 ± 20.1**
6	59.0 ± 3.9	97.9 ± 5.6**
12	107.5 ± 13.9	147.2 ± 23.8
GGT (U/l)		
0	< 5.0	< 5.0
3	< 5.0	< 5.0
6	< 5.0	< 5.0
12	< 5.0	11.4 ± 3.2
TG (mmol/l)		
0	1.08 ± 0.12	1.06 ± 0.05
3	1.03 ± 0.15	1.01 ± 0.18
6	1.14 ± 0.12	1.04 ± 0.08
12	0.89 ± 0.08	1.15 ± 0.08*
CHOL (mmol/l)		
0	< 2.59	< 2.59
3	2.74 ± 0.02	3.28 ± 0.28
6	2.66 ± 0.03	3.11 ± 0.09*
12	2.70 ± 0.06	3.04 ± 0.13*

Values are given as mean ± SEM.
*: Significantly different from control, $p < 0.05$.
**: Significantly different from control, $p < 0.01$.
ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; GGT = gamma-glutamyl transpeptidase; TG = triglyceride; CHOL = cholesterol.

Hydroxyproline content of liver tissue from ethanol-treated rats was significantly increased (Fig. 4). The hydroxyproline content of control livers was slightly higher than of normal healthy rats of the same cohort (data not shown). These results concur with the histopathological observations of the presence of liver fibrosis in both ethanol-treated and control rats.

Table 4. Retinoid levels and retinyl ester composition (% of total retinoids) in liver of ethanol-fed and pair-fed control rats at the time of sacrifice.

	control	ethanol
retinol equivalent (μg/g liver)	4.3 ± 0.6	7.7 ± 1.4
% retinol	0	0
% r. oleate	0	2.7 ± 0.9*
% r. palmitate	62.8 ± 2.2	59.3 ± 1.2
% r. stearate	37.2 ± 2.2	38.0 ± 1.0

Values are given as mean ± SEM.
*: Significantly different from control, $p < 0.05$.

Figure 4. Hydroxyproline concentration and content of the liver of the control and ethanol-treated rats at the time of sacrifice.
*: Values (mean ± SEM) are significantly different from those of the control group ($p < 0.05$).

DISCUSSION

The present study is an extension of experiments in rats on the effects of chronic alcohol administration on the liver under different nutritional conditions. In earlier experiments, we found that life-long alcohol administration to rats maintained on normal lab chow had severe pathological effects on various organs but not on the liver (18). This absence of direct toxicity of alcohol on the liver of rats has been confirmed by others, and is also likely to hold true for monkeys and man. Recently, it was reported from a prospective study of chronic alcoholics that nutritional support had a beneficial effect on the alcohol-induced liver damage (32).

Rao and co-workers concluded from their experimental studies in rats and from a critical review of published studies on alcohol consumption and nutritional conditions in monkeys and man, that alcohol requires a nutritional co-factor to exhibit hepatotoxicity

(19). The nature of this factor is not yet well-defined, and it may not be limited to one particular food constituent or property of the diet. Recent studies by French and co-workers have addressed the issue of malnutrition as a critical factor for alcohol-related liver damage in experiments with dietary manipulations that are comparable to those applied in our study (27,29,31,32). However, their model is rather artificial in that it involves administration of both ethanol and food through permanent intra-gastric catheters. In addition, unscheduled mortality was high, both as a result of surgical complications, and through ethanol intoxication due to very high blood ethanol levels (31). Ethanol administration leads to centro-central septal fibrosis, but only in some of the rats surviving after 6 months of treatment (31).

In an earlier study in rats, we did find a fibrogenic effect of alcohol when its administration was combined with carbon tetrachloride (CCl_4), a known potent hepatoxic agent (11). From other studies with the CCl4-model in rats, we obtained strong evidence that nutritional factors, notably vitamin A, modulates liver fibrosis induced by CCl4 (20,21). In rats with low hepatic retinoid levels, CCl_4-induced liver fibrosis was more pronounced than in rats with sufficient hepatic retinoid levels (21). Low hepatic vitamin A levels, which can be the result of low dietary intake, but also of interference with vitamin A metabolism by agents such as ethanol (22), may be a risk factor for the development of liver fibrosis. The present study was undertaken to create an experimental model of alcohol-induced liver damage with fibrosis which is only dependent on nutritional factors comprising malnourishment.

Rats were fed a fat-rich, calorie-sufficient, but otherwise poly-deficient diet, qualitatively more or less comparable to the usual food among chronic alcoholics (33). Protein content of the diet was only 3% of total caloric intake, of which choline amounted to 16-25 mg/kg body weight/day, as compared to 30-40 mg/kg body weight/day for adequate lab chow recommended by the Academy of Science (23). No oedema, indicative of overt protein deficiency, was observed in either group. Severe choline deficiency is known to induce liver fibrosis in rats (24) and the marginal choline content may contribute to the fibrosis found in the livers of the non-alcohol treated rats. Choline deficiency has also been found in malnourished patients with liver cirrhosis (25,26), but its role in the pathogenesis of fibrosis of human liver is not yet clear.

The liquid diet was very rich in fat which constituted 38% of the total calorie intake. Calorie intake was within normal limits, being 183-278 Kcal/kg/day as compared with 200 Kcal/kg/day for normal rat lab chow (Hope Farms, Woerden, the Netherlands). Still, body weight gradually decreased, and, in spite of isocaloric nutrition, this was more pronounced in the ethanol-treated than in control rats. This has also been described by others (27). Intake of high dietary fat and high alcohol has been associated, both in experimental animals (27) and in humans (28), with liver damage beyond the production of mere steatosis. Steatosis as such may be the effect of high fat intake, protein-calorie

malnutrition or of the combination of the two but not of alcohol alone. In the same rat strain, chronic ethanol administration has also been combined with normal, protein and calorie sufficient, lab chow; no steatosis, or any other liver abnormality was found (18).

In the present study, the addition of alcohol as whisky to the fat-rich protein-deficient diet resulted in an enhancement of liver fibrogenesis as reflected by the hydroxyproline content and the histology of the liver. The critical values for blood alcohol levels and amounts of dietary fat required for induction of liver fibrosis in rats have not yet been defined, but the amount and type of fat in alcohol-containing diets and high sustained blood alcohol levels are likely to be important for the severity of the alcoholic liver damage, including fibrosis (29). Rats in the Tsukamoto-French model of intragastric alcohol infusion developed severe steatosis and necrosis, often with fibrosis of the liver, on a diet containing 25% of total calories as fat and on BAL averaging 353 mg/dl (30). In the present study, the rats developed liver fibrosis on a dietary fat content of 38% of total calories and on blood alcohol levels ranging from 50-126 mg/dl.

The vitamin A content of our diet was low, as were the hepatic retinoid levels of rats of both groups. The lipid droplet content of both fat-storing cells and septal fibroblasts was high in the study of Tsukamoto and French (30), which strongly suggests that the retinoid content their diet was relatively high.

On the one hand, this may have lowered the incidence of fibrosis unintentionally, since excess vitamin A, although possibly augmenting hepatocellular damage, is known to suppress hepatic fibrogenesis (20,34). On the other hand, their conclusions on the role of proliferation of fat-storing cells in septa formation is questionable, because we have found that septal fibroblasts can acquire vitamin A containing lipid droplets after high doses of the vitamin (20).

In summary, these experimental findings confirm the previous experimental and clinical indications that protein- malnutrition with high fat intake contributes to the fibrogenesis of the liver associated with chronic high alcohol consumption. In this respect, malnutrition may even be the triggering factor necessary for the induction of so-called alcoholic liver disease. Next to choline deficiency, altered vitamin A status may modulate liver fibrosis.

These experimental findings may have important nutritional implications for developing, or evident, liver damage associated with chronic alcohol abuse. Nutritional adequacy of the diet may prevent, limit, and perhaps even revert, existing fibrosis of the liver.

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CHAPTER 4

EXPERIMENTALLY INDUCED LIVER FIBROSIS IN THE RAT: THE RELATION TO ALCOHOL-INDUCED LIVER FIBROSIS

Synergism between ethanol and carbon tetrachloride in the generation of liver fibrosis

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SUMMARY

In this study, alcohol-induced histological lesions in a short-term experimental rat model were compared with those characteristic of human alcoholic liver disease. In the rat model used, pretreatment with carbon tetrachloride (CCl_4) for 6 weeks was employed possibly to sensitize the liver for the effects of alcohol and shorten the time of induction of alcoholic liver disease. After 6 weeks of CCl_4 treatment, subsequent maintenance on drinking water containing up to 10 per cent alcohol for 7 weeks potentiated liver fibroplasia as compared with non-alcohol-treated rats. However, steatosis and alcoholic hepatitis, as histological evidence for alcoholic liver disease as seen in humans, were not observed. In non-CCl_4-pretreatment control animals, alcohol administration had no effect on liver histology.

It can be concluded that in the model used, CCl_4 pretreatment sensitizes the liver to increase collagen deposition following alcohol administration, but not to steatosis or alcoholic hepatitis as seen in human alcoholic liver disease. In this experimental set-up, direct metabolic interaction of CCl_4 with alcohol as a cause of the increased fibroplasia can be excluded.

INTRODUCTION

Alcoholic liver disease (ALD) is one of the most prevalent diseases of the Western world, with serious socio-economic impact on society. Much research on ALD in humans, and also in experimental animal models, has been done especially concerning hepatic fibrosis in later stages. However, important questions still remain to be resolved regarding the pathophysiological mechanisms which lead to early alcoholic fibrosis.

In animals, many studies on the effects of ethanol have been done in rats. Although in some histological studies in rats, steatosis, necrosis, and later fibrosis were produced by chronic alcohol administration (1,2), these changes were not dependent on alcohol administration alone. Alcohol administration per se appeared not to be hepatotoxic without additional modulation of the diet. Furthermore, in none of these studies was the typical histological picture of alcoholic hepatitis, as observed in man, seen.

Whether this makes the rat model an inappropriate tool to study human ALD is, however, doubtful, since the aetiology of human ALD is also multifactorial (3) and in humans heavy chronic alcohol consumption may only lead to (end-stage) ALD in a minority of the cases.

In this paper, we report attempts to study the effect of alcohol on developing fibrosis in a short-term experimental model. The results show that, after sensitization of the liver by pretreatment with CCl_4, subsequent alcohol administration enhances liver fibrosis in

all animals of the group but that no other histological evidence of human ALD, viz. steatosis and alcohol hepatitis, is induced.

MATERIALS AND METHODS

Animals

Four groups of eight male BN/BiRij rats, 11 weeks old, weighing 260 ± 27 g (mean ± SD), were kept in individual cages during the entire experiment. An almost constant temperature of about 22 °C, and an almost constant air humidity of about 60 per cent were maintained. The animals were kept on standard laboratory chow which was given *ad libitum*. Fluid intake was recorded daily and body weight weekly.

Treatment of the animals

Induction of liver fibrosis by CCl_4 was accomplished essentially as described by Geerts et al. (4). Two groups of rats were injected subcutaneously twice a week with 200 μl of CCl_4 diluted in paraffin oil (50 per cent v/v) per 100 g body weight for 6 weeks. The two control groups were injected with paraffin oil only. After this treatment schedule was completed, one group of the CCl_4-treated rats and one of the control rats were given ethanol in drinking water, the other groups normal drinking water. The initial concentration of ethanol was 2.5 per cent (v/v) which was increased stepwise to 10 per cent in 2 weeks and maintained at that concentration for another 5 weeks (Table 1).

Liver tissue histology

Under ether anaesthesia, the rats were subjected to total body perfusion with saline and Karnovsky fixative through the aorta. The entire medial liver lobe was excluded from fixation by ligation during preperfusion with saline. This lobe was immediately frozen in liquid nitrogen for biochemical analysis. The perfusion-fixed remainder of the liver was post-fixed in Karnovsky for light microscopy and routinely processed and embedded in paraplast. Four μm thick sections were stained with haematoxylin-phloxin-saphrane (HPS), Gordon and Sweet (for reticulin) to evaluate the liver architecture. Sirius red-F_3BA (5) and Van Gieson Elastica were used for staining of collagen. For Sirius red staining, the mounted sections were first rinsed with SUSA fixative solution (5) (20 min, 20 °C) to suppress non-specific (nuclear) staining.

Hydroxyproline determination

Part of the frozen medial lobe of the liver was freeze-dried overnight. Hydrolysis was performed by adding 5 ml of 6 M HCl to 60 mg of dried tissue followed by incubation at 100 °C for 16 h in screw-capped glass tubes. After cooling, the hydrolysate was filtered

through a 22 μm Millipore filter. Each tube and filter were rinsed with 5 ml of 6 M HCl which was added to the first filtrate. The hydrolysates were stored at -20°C.

Hydroxyproline determination was performed using the method of Woessner (6) modified according to Stegemann and Stalder (7) and Meistovich et al. (8). Briefly, a 100 μl sample of the hydrolysate was added to a mixture of 100 μl of 6 M NaOH and 1.8 ml of 0.3 M NaCl. One ml of 0.05 M chloramine T in citrate-acetate buffer (pH 6.0) in the presence of n-propanol (1.32 M) was added to this mixture. The solution was mixed and incubated for 20 min at room temperature. The incubation was stopped by adding 1 ml of perchloric acid/4-dimethylaminobenzoaldehyde solution in n-propanol. The colour was developed by incubation for 15 min at 60°C. After cooling, the absorption was determined at 550 nm with an Ultraspec 4050 spectrophotometer (LKB, Cambridge).

Hydroxyproline content was calculated from calibration curves, using purified hydroxyproline as the standard.

Table 1. Consumption of alcohol-containing drinking water by CCl_4-treated and control rats.

Period (days)	Alcohol concentration (%)	Treatment group	
		CCl_4	Controls
1- 3	2.5	177 ± 28*	115 ± 9
4- 7	5	139 ± 23*	103 ± 9
8-10	5	119 ± 27*	90 ± 5
11-14	7.5	113 ± 19*	88 ± 7
15-17	10	95 ± 18	91 ± 8
18-21	10	104 ± 17	97 ± 11
22-24	10	95 ± 17	98 ± 8
25-28	10	103 ± 18	99 ± 9
29-31	10	101 ± 13	94 ± 8
32-35	10	90 ± 13	91 ± 16
36-38	10	105 ± 29	96 ± 12
39-42	10	97 ± 11	96 ± 16
43-45	10	97 ± 14	99 ± 10

Animals were treated with CCl_4 or paraffin oil (controls) for 6 weeks prior to alcohol administration.
Values are given as ml of fluid per kg body weight per day ± SD (n = 8).
*Value differs significantly from control value ($P < 0.01$).

Morphometry

The degree of dissection in fibrotic livers was analysed by computer-aided morphometry using a MOP-Videoplan, a microcomputer system with standard Kontron software, a digitizer tablet, and a microscope with a drawing tube. Surface areas of liver parenchyma

within fibrous septa were measured in Sirius red-F_3BA-stained sections. Measurements were taken directly from microscopic images using a red light-emitting diode fitted to the digitizer cursor. The image of the diode was superimposed on the field of view through the drawing tube and measurements were made by tracing the diode spot along fibrous septa, encircling each area of parenchymatous tissue. For each specimen, 30-40 areas were measured.

RESULTS

Alcohol and water intake

The water intake of untreated rats was 100-130 ml per kg body weight per day. During the 2-week period of adaptation to alcohol, the CCl_4-treated rats consumed somewhat more alcohol than the untreated rats. For both groups, the alcohol consumption was relatively stable at 7.5-8.5 g of pure alcohol per kg body weight per day from day 15 onwards, when the animals were given 10 per cent alcohol (Table 1).

Liver histology

The liver sections of all control rats treated with paraffin oil showed normal liver histology; occasionally, a small focus of predominantly round cell inflammatory infiltrate was found in the parenchyma, as can be found in untreated animals of this strain (not shown). Normal liver morphology was also seen in all control rats post-treated with alcohol only (Figs. 1A and 1B).

Six weeks after the start of the CCl_4 treatment, the liver showed diffuse swelling necrosis of the parenchyma, with ballooning of liver cells and occasional acidophilic bodies, and slight inflammatory infiltrate mainly composed of mononuclear cells. There was dissecting fibrosis of the lobules of the slender septa staining positively with Sirius red (Figs. 2A and 2B).

The livers of all rats treated with CCl_4 followed by administration of normal drinking water showed extensive dissecting fibrosis (Fig. 3), but less than immediately after 6 weeks CCl_4 treatment. Collagen bridges connected central and portal areas of the lobule but there was no evidence of cirrhosis. Pericellular fibrosis and necrosis of the parenchyma outside fibrous septa were not observed. Steatosis was absent.

In the rats treated with CCl_4 followed by ethanol administration (Fig. 4), dissecting fibrosis was more pronounced than that in the rats treated with CCl_4 only and was also comparable with rat liver directly after 6 weeks CCl_4 treatment; the liver parenchyma was subdivided into smaller fields, but nodule formation by regeneration or cirrhosis was not observed. Steatosis and necrosis were absent, as were inflammation or foci of alcoholic hepatitis and pericellular fibrosis outside the fibrous septa.

Figure 1. Rat liver after 6 weeks treatment with paraffin oil followed by 7 weeks alcohol-containing drinking water shows normal histology. No necrosis, steatosis, or increased collagen deposition is seen, with a normal pattern of collagen distribution. Staining with Sirius red (b, arrows) is present in the portal tracts, in the wall of central veins, and, very sparsely, as tiny fibres along the sinusoids of the lobules. (a) HPS: (b) Sirius red.

Figure 2. Rat liver after 6 weeks treatment with CCl_4. Severe parenchymal damage with cell swelling, necrosis with occasional acidophilic bodies, and (slight) inflammatory infiltrate, mainly mononuclear cells. Dissecting fibrosis is extensive (b). (a) HPS: (b) Sirius red.

Figure 3. Rat liver after 6 weeks treatment with CCl_4 followed by 7 weeks normal drinking water without alcohol. Diffuse dissecting fibrosis with slender septa is present but less extensive than in Fig. 2. Sirius red.

Figure 4. Rat liver after 6 weeks treatment with CCl_4 followed by 7 weeks alcohol-containing drinking water. Fibrosis is more extensive than that in Fig. 3 but also more extensive than that in Fig. 2, with subdivision of the parenchyma by thicker fibrous septa into smaller fields. However, no regeneration nodules are seen; steatosis and inflammation, as seen in human alcoholic liver disease, are absent. Sirius red.

Morphometrical and biochemical evaluation of liver fibrosis

The degree of liver fibrosis was evaluated by determination of the hydroxyproline content and the degree of dissection by morphometrical analysis. Treatment of control rats with ethanol did not alter the liver hydroxyproline content (Table 2). Rats treated with CCl_4 had a significantly higher hydroxyproline content than the control rats treated with paraffin oil only. In CCl_4-treated rats, the post-treatment with ethanol caused a substantial additional increase in liver hydroxyproline content.

Table 2. Hydroxyproline content of rat liver after sequential treatment with CCl_4 and ethanol.

Treatment	Hydroxyproline content (μg/g wet weight)
Paraffin oil; water	315 ± 64 (7)
Paraffin oil; ethanol	294 ± 64 (7)
CCl_4; water	535 ± 204 (8)*
CCl_4; ethanol	721 ± 164 (8)*, }

Values given are the mean ± SD (n).
Rats were treated with paraffin oil or CCl_4 twice weekly for 6 weeks, followed by maintenance on normal drinking water or water containing ethanol for 7 weeks.
*Differs significantly ($P < 0.005$, Mann-Whitney U-test) from both groups receiving paraffin oil.
} Differs significantly ($P < 0.05$, Mann-Whitney U-test) from the CCl_4-water group.

The morphometric data in Table 3 show that the surface area of liver parenchyma within fibrous septa is much smaller in CCl_4 rats post-treated with ethanol, compared with rats post-treated with water. The liver parenchyma was subdivided into significantly smaller fields by the fibrous septa.

Table 3. Morphometric analysis of the degree of dissection in fibrotic rat liver after CCl_4 treatment with or without sequential treatment with ethanol.

Treatment	Surface area of liver parenchyma within fibrous septa (arbitrary units)
CCl_4; water	21.0 ± 8.5 (5)
CCl_4; ethanol	7.7 ± 3.8 (6)*

Values given are mean ± SD (n).
*Differs significantly ($P < 0.001$, Mann-Whitney U-test) from the CCl_4-water group.

DISCUSSION

The aim of this study was to determine whether histological liver lesions that are considered to be characteristic of human alcoholic liver disease could be elicited in a rat model by alcohol administration.

In rats, several histological effects of ethanol on the liver have been described. The nature and severity of these effects vary with the route of alcohol administration and the composition of the diet, especially regarding fat content (2) and vitamin A supplementation to the diet (1). The histopathological effects of alcohol in rats are comparable to some extent with the effects observed in human alcoholic liver disease. In rats, these effects may include steatosis with or without pericellular fibrosis, swelling and necrosis of hepatocytes with predominantly mononuclear cell infiltrate, and perivenular and dissecting fibrosis. However, the histological picture of human alcoholic hepatitis (9,10) composed of damage of parenchymal cells with Mallory body formation and inflammatory response with mainly polymorphonuclear cells, and pericellular fibrosis, has not been described in a rat model. This highly characteristic histological picture of alcoholic hepatitis in human liver is still generally considered to be the hallmark of progression to cirrhosis (11). However, from experimental studies in primates (12,13) and more recently also from studies in man (14-16), it has been suggested that this characteristic histological picture of alcoholic hepatitis is not the obligatory precursor lesion of progressive alcoholic liver disease leading to cirrhosis.

The possibility remains that alcoholic hepatitis with polymorphonuclear cellular infiltrate in man is only a secondary phenomenon, possibly mediated by leucocytotactic factor(s), which would make it invaluable as a histological prognosticator of progressive ALD.

CCl_4 is known as a potent hepatotoxic agent and as an inducer of hepatic fibrosis (17). As early as 2 weeks after starting CCl_4 treatment, collagen deposition can be observed on light microscopy. In order possibly to sensitize the liver for alcohol and shorten the time span of induction of ALD, we pretreated rats with CCl_4 which resulted in dissecting liver fibrosis but without transition to cirrhosis. When after CCl_4 treatment rats were given ethanol-containing drinking water, the dissecting liver fibrosis was more severe in all animals as compared with the fibrosis in those that received CCl_4 treatment only; this was also reflected by the difference in hydroxyproline content of the liver in both groups. In rats post-treated with alcohol, liver fibrosis was more extensive than directly after CCl_4 treatment. In contrast, post-treatment with normal drinking water resulted in partial recovery. These results indicate that administration of ethanol to CCl_4-treated rats results not only in inhibition of the recovery of the liver but also in a further progression of collagen deposition in fibrous septa. As only histological evidence of LAD (increased) fibrosis was seen; cirrhosis was not observed within the 7-week period of alcohol administration.

Other studies on the combined effects of ethanol and CCl_4 have shown that during simultaneous treatment of rats with these agents ethanol augments the hepatotoxic action of CCl_4 as reflected by serum transaminases (18,19) and liver hydroxyproline content (18). Since, in these studies, ethanol was given from the start of CCl_4 treatment, a direct effect of ethanol on the metabolism of CCl_4 was considered to be the most likely cause for the enhanced toxicity (20,21). In our study, ethanol was shown also to be effective when given after CCl_4, which excludes metabolic interaction as a major determinant.

It can be concluded that CCl_4 pretreatment sensitizes the liver of rats to increased collagen deposition by alcohol administration but not to steatosis or alcoholic hepatitis as seen in human ALD.

This study shows that in a rat model alcohol consumption can be an important determinant of the course of liver fibrosis even in a short-term experiment. This experimental approach appears to be particularly useful for studying the effects of alcohol consumption on liver fibrosis induced by other agents, such as methotrexate and other hepatotoxins.

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CHAPTER 5

THE ROLE OF VITAMIN A IN EXPERIMENTALLY INDUCED LIVER FIBROSIS

A. DUAL ROLE OF VITAMIN A IN EXPERIMENTALLY INDUCED LIVER FIBROSIS

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B. VITAMIN A DEFICIENCY POTENTIATES CCl_4-INDUCED LIVER FIBROSIS IN RATS

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C. BETA-CAROTENE (PROVITAMIN A) INHIBITS CCl_4-INDUCED HEPATIC INFLAMMATION AND FIBROSIS IN RATS

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A. DUAL ROLE OF VITAMIN A IN EXPERIMENTALLY INDUCED LIVER FIBROSIS

ABSTRACT

The effect of vitamin A on carbon tetrachloride (CCl_4) induced liver damage and fibrosis was examined. Vitamin A administered prior to, or during CCl_4-treatment aggrevated the hepatotoxicity of CCl_4, and 6/6 and 4/9 animals respectively died during the CCl_4-treatment. None of the rats died when treated with CCl_4 only. Steatosis and necrosis with inflammation of the liver parenchyma were increased when compared to rats treated with CCl_4 alone. In the rats which were pretreated with vitamin A, liver fibrosis was not observed at the time of death, whereas liver fibrosis was induced in rats which were simultaneously treated with vitamin A and CCl_4. The degree of fibrosis in the livers of the last group was at least as high as in rats treated with CCl_4 only.

On the other hand, vitamin A posttreatment (6 weeks) was not hepatotoxic in rats with CCl_4-induced liver fibrosis. Neither steatosis nor necrosis of the liver parenchyma were observed. Furthermore, vitamin A posttreatment strongly reduced hydroxyproline content of the fibrotic livers when compared to treatment with CCl_4 only. Light microscopy showed that the septa of mature collagen became thin. The fibroblast-like cells in contact with the thin septa were extremely filled with fat droplets containing vitamin A. Electron microscopically, these fibroblast-like cells strongly resembled fat-storing cells. These results further stress the hepatotoxic potential of vitamin A in experimental liver disease and the dual role of vitamin A in liver fibrosis particularly. Depending on the time of administration, vitamin A potentiates parenchymal cell damage and reduce liver fibrosis.

INTRODUCTION

Conflicting results have been reported on the effect of vitamin A administration on experimentally induced liver injury and fibrosis. Studies showed that vitamin A may act synergistically with ethanol in the generation of liver injury and fibrosis (10), while it was also reported to suppress induction of fibrosis by CCl_4 or heterologous serum (13). In this study, vitamin A was administered prior to, during or after CCl_4-treatment. The effect of vitamin A on the induction of liver fibrosis and on an established fibrosis was examined.

MATERIAL AND METHODS

Experimental set up

Female rats of the BN/BiRij strain, aged 3 months, weighing 160 ± 11 g (mean ± SEM, n = 33), were obtained from the institutes colony (Rijswijk, The Netherlands). Rats were maintained under "clean conventional" conditions and were fed a standard laboratory chow ad libitum. Animals were divided into five groups (Fig. 1). Rats of group 1 to 4 received s.c. injections of CCl_4 twice weekly for 4 weeks (200 μl diluted in vegetable oil (1:1 v/v) per 100 g body weight). Rats of group 2 were pretreated with 12.500 IU retinyl palmitate per 100 g body weight in vegetable oil orally twice a week for 4 weeks. The third group was given 12.500 IU retinyl palmitate in vegetable oil per 100 g body weight twice weekly for 4 weeks at the same time the CCl_4 injections were given. The fourth group was posttreated with 50.000 IU of retinyl palmitate in vegetable oil 5 times a week for 6 weeks, started a half week after the last injection of CCl_4. The fifth group was not treated with CCl_4 but received 50.000 IU retinyl palmitate in vegetable oil 5 times a week for 6 weeks.

Figure 1. Experimental set-up of the study. Groups of rats were treated with CCl_4 (1, n = 6), with vitamin A prior to CCl_4-treatment (2, n = 6), with vitamin A and CCl_4 simultaneously (3, n = 9), with vitamin A after CCl_4-treatment (4, n = 6), or with vitamin A only (5, n = 6) (see Materials and Methods).

Surgical liver biopsies

Surgical liver biopsies were taken under halothane anaesthesia at 1 week before CCl_4-treatment and at the end of the CCl_4-treatment (n = 4; five groups). Wedge shaped sections of 40 to 50 mg of the median lobe were removed with an iris scissor (Stöpler, The Netherlands). A part of each liver biopsy was fixed in 4% buffered formalin for light microscopy. The remainder was stored at -70°C until analysis for vitamin A and hydroxyproline. The bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, The Netherlands) and the peritoneum and the abdominal wall were stitched with absorbable surgical suture (4-0 Dexon plus, Davis + Geck, U.S.A.). The skin was cramped with wound clips (Clay Adams, U.S.A.) which were removed one week after surgery.

Liver preparation

Before sacrifice, the animals were fasted overnight. The liver was perfused with PBS through the portal vein for 5 min at 5 ml/min. The median lobe was then ligated, excised, weighed and stored at -70 °C until biochemical analysis. The left lateral lobe was temporarily excluded from perfusion by a lose ligation. The right lateral and the caudate liver lobe were then fixed by perfusion with 2.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 5 ml/min for 8 min) for electron microscopy. The ligation of the left lateral lobe was removed and this lobe was then perfusion fixed with 4% buffered formalin for light microscopy.

Hydroxyproline determination

Liver collagen content was measured by hydroxyproline determination. 30 to 40 mg of liver biopsy or 300 to 500 mg of the frozen median lobe of the liver were lyophilized overnight. The lyophilized liver was powdered and subsequently extracted 5 times with 2 ml or 20 ml diisopropylether, respectively, to remove vitamin A, which suppresses the colour development in the assay. Traces of diisopropylether were evaporated at 37 °C. Subsequently, the lyophilized tissue was hydrolysed by 5 ml 6 N HCL per 300 mg wet weight for 16 h at 120 °C. The hydrolysate was filtered through a 0.22 μm Millipore filter. The hydrolysates were stored at -20 °C. Hydroxyproline determination of the hydrolysates was performed as described earlier (1). Results were expressed as μg hydroxyproline/ g liver tissue or μg hydroxyproline/total liver.

Light and electron microscopy

For light microscopy, 4 μm sections were cut from paraplast embedded tissue. Sections were stained with haematoxylin-phloxin-saphrane (HPS) and Sirius-red F3BA for collagen (8). For electron microscopy, representative samples were taken from the right lateral and the caudate lobe which were postfixed in 1% osmium tetraoxide, dehydrated in graded concentrations of ethanol and embedded in epon LX 112 (Ladd Research Industry Inc., Burlington, VT). Ultrathin fibratome sections were mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined under an EM 410 electron microscope (Philips, Eindhoven, The Netherlands).

Biochemical analysis of blood

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyl transaminase (GGT) were measured in heparin-blood by the Reflotron system (Boehringer Mannheim, F.R.G.) both at the time a liver biopsy was taken and at the time of sacrifice.

RESULTS

No animals died after CCl_4-treatment alone (group 1). However, when CCl_4 was preceded by or given simultaneously with vitamin A, 6/6 and 4/9 rats respectively died in the third week of CCl_4-treatment (groups 2 and 3). None of the rats died when vitamin A was administered after CCl_4-treatment (group 4).

Histological evaluation of the rat livers of groups 2 and 3 showed severe steatosis and parenchymal cell necrosis in both portal and central areas and also a severe infiltration of polymorphonuclear and round nuclear blood cells (Figs. 2B and C). The lesions were more severe than those observed in livers of rats treated with CCl_4 only (Fig. 2A), in which parenchymal cell necrosis mainly occurred close to the central veins. ALAT, ASAT

Figure 2. Rat liver after 3 weeks of CCl_4-treatment. (A1,2) Parenchymal cell swelling, necrosis and inflammatory infiltrate is seen. Diffuse dissecting fibrosis is present. (B1,2) After vitamin A pretreatment, lesions in the liver parenchyma are more severe than after CCl_4-treatment only, but no fibrosis is seen. (C1,2) Vitamin A simultaneously administered with CCl_4-treatment also enhance CCl_4 toxicity in the liver parenchyma. In contrast to vitamin A pretreatment, extensive fibrosis with septa is present. (1) HPS; (2) Sirius red; (PT) portal tract; (CV) central vein.

Table 1. ASAT, ALAT and GGT (IU/L) activities in blood

Group	1	2	3	4	5
Before CCl_4					
ASAT	33 ± 1	37 ± 1	43 ± 4	44 ± 2	30 ± 3
ALAT	78 ± 5	97 ± 4	86 ± 5	94 ± 6	76 ± 4
GGT	< 5	< 5	< 5	< 5	< 5
	n = 6	n = 6	n = 6	n = 6	n = 6
After 3 weeks CCl_4					
ASAT	409 ± 55	1565 ± 177*	440 ± 58	389 ± 45	-
ALAT	1785 ± 254	4600 ± 165*	2035 ± 184	1623 ± 174	-
GGT	23 ± 1	29 ± 7	34 ± 2	21 ± 1	-
	n = 6	n = 3	n = 5	n = 6	
Sacrifice					
ASAT	49 ± 3	x	48 ± 3	37 ± 2	42 ± 3
ALAT	94 ± 3	x	98 ± 5	74 ± 3	93 ± 4
GGT	< 5	x	< 5	< 5	< 5
	n = 6		n = 5	n = 6	n = 6

Values are given as mean ± SEM. Significance was tested with the Student t-test.
*: Significantly higher than after CCl_4-treatment only ($p < 0.05$).
-: No CCl_4-treatment.
x: Rats died spontaneously.

and GGT activities in blood of rats of groups 2 and 3 were more elevated than in those of group 1 (Table 1). Acute liver failure is most probably the cause of death of the animals of groups 2 and 3.

Liver fibrosis was not observed in animals of group 2 (Fig. 2B). Also, liver hydroxyproline content remained at the level of untreated control rats (Table 2). In group 3, both the animals which died spontaneously and those which survived had developed dissecting fibrosis with connective tissue septa staining for collagen (Fig. 2C). At the time of sacrifice, the hydroxyproline content of these livers was comparable to the livers of the CCl_4 treated animals (group 1; Table 2). In livers of animals of both group 1 and 3, connective tissue septa connected central with portal areas of the liver lobule (Figs. 2A and C). Only septa formation and no pericellular parenchymal fibrosis was observed.

The rats which were posttreated with vitamin A (group 4), received a much higher dose than animals of groups 2 and 3 (total dose of 1.5×10^6 and 1.6×10^5 IU, respectively). In spite of the high dose, no signs of toxicity were observed. Neither steatosis nor parenchymal cell necrosis was observed at the time of sacrifice (Fig. 3B).

Table 2. Hydroxyproline content in liver

Group	1	2	3	4	5
Before CCl_4					
HP μg/g liver	117 ± 20	89 ± 24	149 ± 40	127 ± 24	98 ± 14
	n = 4	n = 4	n = 4	n = 4	n = 4
After 3 weeks CCl_4					
HP μg/g liver	413 ± 122	116 ± 60*	485 ± 98	418 ± 157	-
	n = 4	n = 3	n = 4	n = 4	
Sacrifice					
HP μg/g liver	414 ± 138	x	339 ± 72	243 ± 73[&]	132 ± 37
HP μg/liver	2943 ± 1128	x	2343 ± 578	1968 ± 610[&]	1051 ± 243
	n = 6		n = 5	n = 6	n = 6

Values are given as mean ± SEM. Significance was tested with the Student t-test.

*: Significantly lower than three weeks CCl_4 only ($p < 0.05$).

-: No CCl_4-treatment.

x: Rats died spontaneously.

[&]: Significantly lower than after CCl_4-treatment only ($p < 0.05$).

Figure 3. Rat liver after 4 weeks of CCl_4-treatment followed by 6 weeks of no treatment (A1,2) or followed by 6 weeks of vitamin A administration (B1,2). In both livers, neither steatosis nor necrosis of the parenchyma is present. Regression of the fibrotic septa is seen after vitamin A post-treatment (B2). (1) HPS; (2) Sirius red; (PT) portal tract; (CV) central vein.

Blood ALAT, ASAT and GGT activities were normal. Histological examination showed only thin connective tissue septa (Fig. 3B). The hydroxyproline content was significantly lower than in the livers of rats treated with CCl_4 only (Table 2). The FSC in the liver parenchyma as well as the fibroblast-like cells present in the septa were filled with lipid droplets. Cisternae of the rough endoplasmic reticulum were not swollen in either cell type, suggesting that the synthesis of secretory proteins, such as collagens, was relatively low. The morphology of the fibroblast-like cells resembled that of FSC (Fig. 4).

Figure 4. Electron micrograph of a liver after 4 weeks CCl_4-treatment followed by 6 weeks of vitamin A administration, showing a connective tissue septum (S). Fibroblast-like cells (F), which resemble fat-storing cells, are in contact with the septum. In the CCl_4 treated groups bile duct cell (B) proliferation is sometimes observed. (K) Kupffer cell (1210x; inset 2530x).

DISCUSSION

In these experiments, we observed a dual effect of vitamin A on the hepatoxicity of CCl_4 which was related to the time of administration: 1) Vitamin A enhanced the hepatoxicity of CCl_4 when administered prior to or during CCl_4-treatment. Significant mortality was found in contrast to CCl_4-treatment alone. 2) vitamin A was not hepatotoxic when administered after the CCl_4-treatment. Furthermore, administration of high doses resulted in an enhanced removal of CCl_4 induced fibrotic septa. These results are partly in contrast with those reported by Senoo (13). Although suppressional effects of vitamin A on the induction of liver fibrosis were observed, however, toxicity due to vitamin A administration in combination with CCl_4 was not mentioned. No mortality was reported.

The apparent discrepancy between our results and those of Senoo may be the result of minor differences in treatment schedules or strain differences.

The mechanism of enhanced toxicity of vitamin A in combination with CCl_4 is not known. CCl_4 is hepatotoxic through the formation of free radicals by cytochrome P-450 in the liver endoplasmic reticulum (5,15). It has been shown that chronic excess of vitamin A also can be hepatotoxic (4,9,12). Vitamin A administration may produce moderate changes in parenchymal cells, such as endoplasmic reticulum proliferation and enlargement of mitochondria (10). It was proposed that vitamin A is metabolized by liver microsomes, especially when cytochrome p-450 activity is stimulated by hepatotoxic agents such as ethanol (11). The produced vitamin A metabolites might play a role in the increased hepatotoxicity of CCl_4.

Other studies showed that high doses of vitamin A stimulate Kupffer cell function in vivo (2,7). Hypervitaminosis A enhanced the plasma clearance of colloids and led to changes in Kupffer cell morphology suggestive for Kupffer cell activation (2). Plasma clearance of endotoxin was more rapid than in normal rats, but small doses of endotoxin which caused only minimal parenchymal cell injury in normal rats, produced significantly more parenchymal cell necrosis in hypervitaminosis A rats. It was demonstrated that activated Kupffer cells produce mediators which induce cell damage (7). The mechanism of both enhanced toxicity as well as a decreased liver fibrosis might be related to Kupffer cell activation. Enhanced toxicity of vitamin A in the liver during CCl_4-treatment may activate Kupffer cells resulting in an enhanced mediator production leading to an increased cell damage. An enhanced production of acute phase reactants by Kupffer cells as a consequence of activation both by vitamin A and by the uptake of necrotic material in the damaged liver, may lead to a more severe damage of the liver parenchyma. However, in the absence of CCl_4, Kupffer cell activation by excess vitamin A may have a beneficial effect by stimulating collagen breakdown in fibrotic septa.

The suppressional effect of vitamin A on the induction of liver fibrosis by CCl_4 (group 2) and the effect on the reduction of liver fibrosis (group 4), may be due to influences of vitamin A on the metabolism of FSC in normal liver and of both FSC and fibroblast-like cells in the fibrotic liver. In normal liver, orally administered vitamin A is transported to and stored in the FSC (6). Our study shows that, in fibrotic livers, newly administered vitamin A can also be stored in fibroblast-like cells which provides new evidence for the idea that these cells are derived from FSC. Stored in these cells, vitamin A may reduce their collagen synthesis, as was seen in cultured FSC which were loaded with vitamin A before isolation (14). Suppression of the induction of liver fibrosis did not occur when vitamin A was administered simultaneously with CCl_4 (group 3). This suggests that CCl_4 may prevent the simultaneously added vitamin A to accumulate in the FSC and fibroblast-like cells.

Several studies have shown that the fibroblast-like cells associated with fibrotic septa

may be derived from FSC by migration, transformation and cell division (3,16). Our study shows that these fibroblastic cells have, indeed, the capacity to store large amounts of vitamin A. Furthermore, the storage of vitamin A alters their ultrastructural morphology to make it very similar to that of FSC. These findings further support the involvement of FSC in the formation of fibrotic septa.

In conclusion, a high level of vitamin A in FSC and fibroblastic cells in fibrotic septa probably suppresses the potential of these cells to contribute to fibrogenesis of the liver. However, vitamin A may potentiate the hepatotoxicity of agents such as CCl_4 through metabolic interactions in parenchymal cells or by affecting the reactions of Kupffer cells.

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B. VITAMIN A DEFICIENCY POTENTIATES CCl_4-INDUCED LIVER FIBROSIS IN RATS

SUMMARY

Earlier studies have shown that retinoid administration suppresses the generation of hepatic fibrosis and stimulates its regression in normal (i.e. vitamin A sufficient) CCl_4-treated rats. The present study focuses on the possible role of a marginal or deficient vitamin A status on CCl_4-induced fibrosis.

This experimental study in rats shows that vitamin A status, reflected by hepatic retinoid content (retinol and retinyl esters), modulates the development of hepatic fibrosis induced by CCl_4. In rats with low hepatic retinoid levels (12 ± 0.9 μg/g liver), CCl_4-induced liver fibrosis was more pronounced than in rats with sufficient hepatic retinoid levels (1065 ± 327 μg/g liver). Enhanced liver fibrogenesis was confirmed both morphologically and by a higher hydroxyproline content of the liver. It was associated with a reduced liver weight and the development of parenchymal regeneration nodules. Furthermore, CCl_4-treatment itself reduced the hepatic retinoid content in rats independently of the liver vitamin A status before treatment and increased serum retinol levels in vitamin A sufficient rats.

The results show that the vitamin A status of the liver plays an important role in hepatic fibrogenesis. Low hepatic vitamin A levels, which can be the result of low dietary intake, but also of interference with vitamin A metabolism by agents such as ethanol and CCl_4, may be a risk factor for the development of liver fibrosis. We suggest that retinoids modulate collagen synthesis and deposition irrespective of the degree of hepatocellular necrosis induced by CCl_4. The reduction of retinoid levels in fat-storing cells and fibroblast-like cells by an enhanced secretion of retinol from the liver into the circulation during CCl_4-treatment, may stimulate the transformation of these cells to fibroblasts and, in this way, contribute to fibrogenesis of the liver.

INTRODUCTION

Hepatic fibrosis, or excess deposition of collagen in the liver, is a common lesion of most clinically-relevant chronic liver diseases (1). Hepatic fibrosis is usually related to past or ongoing inflammation of the liver and may eventually constitute an irreversible process with diffuse parenchymal nodular transformation and cirrhosis. Excess collagen in the liver in itself may cause serious clinical problems such as liver dysfunction and portal hypertension. However, the cellular and molecular basis of the processes underlying this

excessive accumulation of extracellular matrix in the liver is still largely unknown.

The liver is the most important organ in homeostasis, metabolism and storage of retinoids. The parenchymal liver cells play a major role in the primary uptake and processing of retinoids, whereas fat-storing cells are the main storage sites for retinoids in rat liver (2-6). Recent results from our laboratory (7) and previous results obtained by Senoo and co-workers (8) indicate that retinoid supplementation, which increases the liver retinoid stores, modulates the induction of liver fibrosis by CCl_4 in rats. Retinoids administered before, during or after CCl_4-treatment suppressed the induction or enhanced the regression of hepatic fibrosis (7). However, high doses of retinoids administered prior to, or during, CCl_4-treatment enhanced the direct hepatotoxicity of CCl_4, as reflected by an inflammatory response with high serum transaminases, and were associated with the unexpectedly-early death of the animals (7).

In the present study we examined the effects of liver retinoid status on the induction of experimental liver fibrosis. To avoid aggravated hepatotoxicity by the administered retinoids during CCl_4-treatment, as shown in the previous study (7), three groups of rats were with a) sufficient, b) marginal and c) low liver retinoid levels prior to the administration of CCl_4. The data presented indicate that a low liver retinoid content (retinol and retinyl esters) at the time of induction of liver fibrosis by CCl_4 accelerates the development of liver fibrosis and increases hepatic collagen deposition.

MATERIAL AND METHODS

Experimental design

Three-week-old female, specific pathogen-free, inbred WAG/MBL rats, weighing 54 ± 4 g (mean ± SEM, n = 29), were obtained from the TNO Medical Biological Laboratory (Rijswijk, the Netherlands). Three animals were sacrificed as controls for histological examination and for biochemical analysis of the vitamin A and hydroxyproline content of normal untreated liver. At the start of the experiment, the liver of the weanling rats contained a retinoid level (retinol and retinyl esters) of 86.6 ± 13.3 μg retinol equivalents per g liver (n = 3).

In order to obtain rats with different liver vitamin A status but without differences in other respects, the following plan was followed. The rats (n = 26) were housed in Makrolon type-3 cages (two per cage) and were maintained under clean conventional conditions (9). Water and a commercial pelleted semi-synthetic diet (Hope Farms, Woerden, the Netherlands) were supplied ad libitum. To reduce the liver retinoid stores, the chow was deficient for retinoids other than retinoic acid, which cannot be converted by the body into storage forms of vitamin A. Retinoic acid was added to the diet (2.0 mg per kg) to avoid the development of signs of vitamin A deficiency (10). The rats were

kept on the diet until sacrifice. Body weight was measured weekly. After a period of 15 weeks, surgical liver biopsies were taken to characterize the retinoid status of the liver. At this time, liver retinoid levels were low (14.1 ± 1.2 μg retinol equivalents per g liver (n = 6)), but none of the rats showed any of the signs of vitamin A deficiency that are characteristic for this strain (10).

For sake of convenience, we designated three groups of animals as rats with a sufficient, marginal and low liver vitamin A status. The rats were randomly divided into three groups. Mean body weight of the three groups was similar. At week 16, rats of group 1 (n = 6) and group 2 (n = 8) were administered two intragastric doses of 30.000 and 3.500 IU retinyl palmitate respectively in sunflower oil, to elevate liver retinoids to sufficient and marginal levels, respectively (6). Rats of group 3 (n = 12) received vitamin A-free sunflower oil, to maintain low liver retinoid levels. In week 17, the vitamin A and hydroxyproline content of the liver were determined (n = 3). From week 18 until sacrifice, all rats received subcutaneous injections of 200 μl CCl_4 diluted in vegetable oil (vitamin A deficient sunflower oil; 1:1 v/v) per 100 g body weight twice weekly (on Tuesday and Friday). In week 22, liver biopsies were taken from some rats (n = 3 per group). Half of each group was sacrificed in week 24, after 6 weeks of CCl_4-treatment. The other half was sacrificed two weeks later, after 8 weeks of CCl_4-treatment.

Surgical liver biopsies

Surgical liver biopsies were taken to determine hepatic vitamin A and hydroxyproline content and to assess liver morphology in the course of the experiment. The rats were anaesthesized by halothane, and wedge-shaped sections of 50 to 75 mg were removed from the median lobe with an iris scissor (Stöpler, Utrecht, the Netherlands). A part of each liver biopsy was fixed in 4% buffered formalin for light microscopy. The remainder (50-60 mg) was stored at -70°C for later analysis of retinoid and hydroxyproline content. The bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, the Netherlands) and the peritoneum and the abdominal wall were sutured with absorbable surgical material (4-0 Dexon plus, Davis + Geck, Manati, USA). The skin was closed with wound clips (Clay Adams, Parsippany, USA), which were removed one week after surgery.

Liver preparation and histopathology

The rats were fasted overnight before sacrifice to deplete the liver of glycogen and this facilitate electron microscopical examination. At sacrifice, liver tissue was collected after perfusion with phosphate buffered saline (PBS, pH 7.4) through the portal vein for 5 min at 5 ml/min, followed by perfusion fixation. The median lobe was excluded from fixation by ligation and, after removal from the body, it was weighed and immediately frozen in liquid nitrogen and stored at -70°C for later biochemical analysis. The left lateral lobe

was temporarily excluded from perfusion by application of a loose ligature. The right lateral and the caudate liver lobe were then fixed by perfusion with 2.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4, 5 ml/min for 8 min) and processed for electron microscopy. The ligature of the left lateral lobe was removed and this lobe was then fixed by perfusion with 4% buffered formalin for light microscopy. After fixation, the liver was removed from the body and weighed.

For light microscopy, liver tissue was routinely processed and embedded in paraplast. Sections, four micron thick, were stained with haematoxylin-phloxin-saffron (HPS) or Sirius-red F_4BA for collagen. The assessment of morphology was performed by a pathologist who was working blind to the specific treatment of the liver. The sections of liver biopsies and of post mortem liver tissue were examined for the presence of inflammation, fibrosis and cirrhosis. The degree of liver fibrosis was analysed by morphometry.

Morphometry

The degree of dissection of the livers by fibrosis was analysed by computer-aided morphometry using a MOP-Videoplan, a microcomputer system with standard Kontron software, a digitizer tablet, and a microscope with a drawing tube. Surface area of liver parenchyma within fibrous septa was measured in Sirius-red F3BA-stained sections. Measurements were taken directly from microscopic images using a red light-emitting diode fitted to the digitizer cursor. The image of the diode was superimposed on the field of view through the drawing tube and measurements were made by tracing the diode spot along fibrous septa, encircling each area of parenchymatous tissue. Each area was expressed in arbitrary units. Low units were obtained when small parenchymal cell areas were measured indicating a severe dissecting fibrosis. For each specimen, 30-40 areas were chosen at random and measured. For each liver, the average surface area was calculated. Group means were expressed as the mean ± SEM.

Electron microscopy

For electron microscopy, samples were taken randomly from the right lateral and the caudate liver lobe of rats sacrificed after 8 weeks of CCl_4-treatment. The samples were postfixed in 1% osmium tetroxide, dehydrated in graded concentrations of ethanol and embedded in epon LX 112 (Ladd Research Industry Inc., Burlington, VT, USA). Ultrathin fibratome sections were mounted on uncoated copper grids, contrasted with uranyl acetate and lead citrate, and examined with an EM 410 electron microscope (Philips, Eindhoven, the Netherlands).

Retinoid analysis

The content of retinol and the three main retinyl esters, viz. retinyl palmitate, retinyl

stearate and retinyl oleate, were determined in liver and plasma using high performance liquid chromatography (HPLC) (2).

Hydroxyproline determination

Liver collagen content was measured by hydroxyproline determination. Samples of liver biopsies (30 to 40 mg) or of the frozen median lobe of the liver (300 to 500 mg) were lyophilized overnight. This lyophilized liver tissue was powdered and subsequently extracted 5 times with 2 ml or 20 ml diisopropylether, for biopsies and post mortem liver respectively, to remove vitamin A which suppresses the colour development in the assay (7). Traces of diisopropylether were evaporated at 37°C. Subsequently, the lyophilized tissue was hydrolysed by 5 ml 6 N HCL per 300 mg wet weight for 16 h at 120°C. The hydrolysate was filtered through a 0.22 μm Millipore filter. The hydrolysates were stored at -20°C. Hydroxyproline determination of the hydrolysates was performed as described previously (11).

Biochemical analysis of blood

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyl transpeptidase (GGT) were measured in heparinized blood by the Reflotron system (Boehringer Mannheim, Mannheim, Germany), both at the time of liver biopsy and at sacrifice.

Statistics

Values are expressed as the mean ± SEM. Group means of the test groups were evaluated for statistically-significant differences using the Mann-Whitney U test (significance levels 5%, 1% and 0.1%).

RESULTS

Retinoid content of the liver

Prior to CCl_4 treatment, the retinol and retinyl ester concentrations in the livers of rats of group 2 (marginal) and 3 (deficient) were 4 and 90 times lower, respectively, than of group 1 (sufficient) (Fig. 1A).

During the period of CCl_4-treatment, the retinol and retinyl ester concentration of the livers of all groups were reduced (Fig. 1A). After 6-8 weeks of treatment, the concentration in group 1 and 2 was decreased to about 25 % of the pre-treatment values. The already low retinol and retinyl ester concentration of livers of group 3 were further dropped to very low levels.

Figure 1. Effect of CCl_4-treatment on retinoid levels in liver and plasma of rats with different vitamin A status.
Three groups of rats, with sufficient, marginal and low liver retinoid contents were treated with CCl_4 during week 18-26 (see Material and methods). Rats were sacrificed at week 17, 24 and 26. Total retinoid contents of the liver (a), and retinol levels in plasma (b) were determined as described in Material and methods. Values are given as the mean ± SEM. * Significantly different ($p < 0.05$) from values at week 17 (a) or 24 (b).

In rats of all groups, the retinol and retinyl ester composition of the liver altered as a result of CCl_4-treatment (Table 1). In groups 1 and 2, a selective loss of palmitate ester was noted in favour of the oleate and stearate esters, while the percentage of retinol was not changed in either group. Although the absolute retinol liver levels were very low in group 3, relative retinol levels were higher (Table 1), which is normal for retinoid deficiency (10).

Plasma retinol levels (Fig. 1B) were affected by CCl_4-treatment. In groups 1 and 2, the levels were 5-10 times higher than those found in vitamin A sufficient rats of the same cohort not treated with CCl_4 (17 ± 2 µg/dl plasma). Plasma retinol levels in rats of group 3 were 30-40% of those in normal vitamin A sufficient rats.

Biochemical and histological parameters of liver fibrosis

Post mortem macroscopical examination after 8 weeks of CCl_4-treatment showed coarse parenchymal nodules on the surface of the livers of rats of group 2 and particularly of group 3 (Fig. 2B). The livers of group 1 showed only a few parenchymal nodules on the surface, and were not cirrhotic (Fig. 2A). The liver weight and the ratio liver/body weight of rats from group 2 and 3 were significantly lower than those in rats of group 1 (Table 2). Similar results were found at 6 weeks (not shown).

Table 1. Relative composition of retinoids in liver of rats with sufficient, moderate and low retinoid liver status before treatment.

week	17*	24 (6)*	26 (8)*
Sufficient:			
% retinol	1.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
% r. oleate	8.5 ± 0.8	9.3 ± 0.1	10.7 ± 0.6**
% r. palmitate	82.2 ± 0.6	77.8 ± 0.7	70.8 ± 0.6**
% r. stearate	7.9 ± 0.3	12.2 ± 0.6	17.8 ± 0.3**
	n = 3	n = 3	n = 3
Moderate:			
% retinol	3.1 ± 0.9	5.0 ± 0.4	2.9 ± 0.7
% r. oleate	9.1 ± 0.3	8.2 ± 0.3	10.8 ± 0.6
% r. palmitate	79.0 ± 0.8	75.5 ± 4.9	66.3 ± 1.3**
% r. stearate	8.8 ± 0.3	11.3 ± 5.0	20.0 ± 0.8**
	n = 3	n = 4	n = 4
Low:			
% retinol	14.3 ± 1.2	48.4 ± 16.4	59.7 ± 8.6**
% r. oleate	2.5 ± 1.5	0.9 ± 0.9	nd
% r. palmitate	61.6 ± 2.9	24.9 ± 12.3	22.8 ± 1.9**
% r. stearate	21.6 ± 0.8	11.1 ± 3.8	17.4 ± 7.1
	n = 3	n = 6	n = 6

* The first number denotes weeks after the rats were put on the diet. Number in parenthesis represents the weeks of CCl_4-treatment (see Materials and Methods). Data are expressed as percentage of total retinoid content of the liver and values are given as the mean ± SEM (n = denotes number of rats)
** Significantly different from values at week 17 ($p < 0.05$).
nd not detectable

Figure 2. Macroscopical view of the liver of rats with sufficient (a) and low (b) vitamin A status after 8 weeks of CCl_4-treatment.
Figure (a) shows a representative example of a fibrotic liver induced by CCl_4 treatment of a vitamin A sufficient rat. The liver is pale and only minor nodule formation is visible at the surface. The liver of rats with low vitamin A status (b) has become cirrhotic and shows many regeneration nodules. The size of the liver is reduced as compared with the vitamin A sufficient rats. An arrow indicates the site of the liver biopsy taken during the course of the experiment (see Material and methods).

Table 2. Body weight, liver weight and percentage liver per body weight of the rats with sufficient (group one), moderate (group two) and low (group three) liver retinoid levels just before and during CCl_4 treatment.

week[a]	body weight (g)[b]	liver weight (g)[b]	% liver per body weight[b]
Sufficient			
18 [0]	185 ± 6 (6)		
22 [2]	188 ± 6 (6)		
24 [6]	185 ± 4 (6)	11.6 ± 0.4 (3)	6.3 ± 0.1 (3)
26 [8]	204 ± 9 (3)	13.2 ± 1.3 (3)	6.5 ± 0.4 (3)
Moderate			
18 [0]	183 ± 5 (8)		
22 [2]	181 ± 5 (8)		
24 [6]	181 ± 7 (8)	10.3 ± 0.2* (4)	5.7 ± 0.2* (4)
26 [8]	192 ± 7 (4)	9.8 ± 0.7* (4)	5.1 ± 0.2* (4)
Low			
18 [0]	179 ± 3 (12)		
22 [2]	175 ± 3 (12)		
24 [6]	180 ± 6 (12)	9.6 ± 0.4* (6)	5.4 ± 0.2* (6)
26 [8]	183 ± 2* (6)	8.8 ± 0.2** (6)	4.8 ± 0.1* (6)

[a] The first number denotes weeks after the rats were put on the diet. Number in brackets represents the weeks of CCl_4-treatment (see Materials and Methods).

[b] Values are given as the mean ± SEM. The number in parenthesis denotes the number of rats.

* Significantly different from group 1 ($p < 0.05$).

** Significantly different from group 1 ($p < 0.01$).

Liver morphology showed fibrosis in all groups but to a different extent. Liver fibrosis was more severe in group 3 (Fig. 3B) than in group 1 (Fig. 3A), as was also indicated by a higher hydroxyproline content (Fig. 4) and by a smaller average surface area of liver parenchyma within fibrous septa (Fig. 5). An intermediate degree of fibrosis was observed in group 2 as compared with group 1 and 3 (not shown). Blood parameters reflected the presence of parenchymal damage in all groups (Table 3). Starting at 8 weeks, ALAT, ASAT and GGT activities in rats of group 3 were significantly higher than those of group 1. Inflammation was confirmed by microscopical examination (Fig. 3C and 3D). Extensive parenchymal cell swelling, foci of parenchymal necrosis and mixed inflammatory cell infiltration were observed in the inflammated areas. In the livers of group 3 particularly, fibroblast-like cells were observed, not only in septa, but also in foci of parenchymal cell necrosis (Fig. 3D). These cells were often closely associated with thin collagen fibres.

Figure 3. Lightmicroscopical analysis of CCl_4-induced liver fibrosis in rats with sufficient (a,c) and low (b,d) vitamin A status.

Light micrographs of the liver after 8 weeks of CCl_4-treatment (week 26). The sections, stained for collagen with Sirius red (a,b), show fibrosis in both groups, but to a different extent. Note that excess collagen deposition is much more pronounced in livers with low retinoid levels (b,d) than in those with sufficient levels (a,c). In addition, more extensive dissection of the liver parenchyma by fibrous septa, and more regeneration nodules are present, and, more fibroblast-like cells were observed at the foci of parenchymal cell necrosis (arrow) in rats with low vitamin A status (d) than in vitamin A sufficient ones (c). Steatosis and severe parenchymal damage with cell swelling, necrosis and mixed inflammatory infiltrate is seen in livers of both groups (c,d). (a,b: Sirius red staining, 10x; c,d: HPS staining, 163x).

Figure 4. Effect of CCl_4-treatment on the hydroxyproline content of the livers of rats with sufficient, marginal and low vitamin A status.
Values are given as the mean ± SEM.
* Significantly different from values of the vitamin A sufficient rats ($p < 0.05$).

Figure 5. Morphometric analysis of the degree of dissecting liver fibrosis after 8 weeks of CCl_4-treatment of rats with different vitamin A status.
Values are given as the mean ± SEM (see material and methods).
* Significantly different from values of the vitamin A sufficient rats ($p < 0.05$).

Ultrastructural examination

Ultrastructural observations after 8 weeks of CCl_4-treatment revealed thin septa in the livers of rats in group 1, with collagen fibrils arranged into large, regularly-formed bundles interspersed with fibroblast-like cells (Fig. 6A). The collagen bundles filled the extracellular space and were arranged close to fibroblasts-like cells. Dilated strands of rough endoplasmatic reticulum (RER) were present in these fibroblast-like cells. Electron-dense bodies, suggestive of lysosomes, varied in size and were always present (Fig. 6B). Lipid droplets and myogenic characteristics, such as distorted shaped nuclei,

Table 3. Values of some blood parameters of liver damage (ALAT, ASAT and GGT) of rats with sufficient (group one), moderate (group two) and low (group three) liver retinoid levels just before and during CCl_4 treatment.

week	14*	17*	19 (1)*	22 (4)*	24 (6)*	26 (8)*
Sufficient						
ALAT	40 ± 2	33 ± 2	369 ± 134	143 ± 5	110 ± 11	104 ± 28
ASAT	83 ± 13	65 ± 3	966 ± 369	430 ± 32	451 ± 44	388 ± 160
GGT	nd	nd	5.1 ± 0.1[d]	8.7 ± 1.0[c]	18.7 ± 10.5[b]	17.6 ± 8.4[a]
	n = 6	n = 6	n = 6	n = 6	n = 6	n = 3
Moderate						
ALAT	41 ± 4	35 ± 3	557 ± 52	210 ± 97	105 ± 19	102 ± 9
ASAT	85 ± 10	66 ± 4	1503 ± 496	503 ± 145	863 ± 237	335 ± 62
GGT	nd	nd	6.8 ± 0.9[c]	16.0 ± 1.9[b]	13.5 ± 8.5[a]	12.9 ± 2.5[a]
	n = 8	n = 8	n = 8	n = 8	n = 8	n = 4
Low						
ALAT	36 ± 3	64 ± 10**	394 ± 72	100 ± 15	180 ± 46	215 ± 67**
ASAT	75 ± 8	100 ± 22**	1311 ± 414	545 ± 139	934 ± 302	691 ± 165**
GGT	nd	nd	nd	9.3 ± 3.2[b]	11.1 ± 2.0[a]	31.8 ± 6.8**
	n = 12	n = 12	n = 12	n = 12	n = 12	n = 6

nd not detectable; all values below the detecion limit of 5 IU/l.

[a] one value below the detection limit was not included.

[b] two values below the detection limit were not included.

[c] three values below the detection limit were not included.

[d] four values below the detection limit were not included.

* The first number denotes weeks after the rats were put on the diet. Number in parenthesis represents the weeks of CCL_4-treatment (see Materials and Methods). Values are given as mean ± SEM.

** Significantly different from group 1 ($p < 0.05$).

ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; GGT = gamma glutamyl transpeptidase.

subplasmalemmal densities and remnants of basal membrane, were inconspicuous and often absent. FSC in the perisinusoidal space revealed an activated appearance as judged by the presence of some short dilated strands of RER and lysosomes. Lipid droplets were regularly seen (Fig. 6C). In contrast with livers of rats in group 1, the septa of livers of rats containing low liver retinoid levels (group 3) were differently organized. The matrix between the slender fibroblast-like cells contained only a few focal collections of collagen fibrils (Fig. 7A). Most of the space was electron lucent or was filled with fuzzy material. The long processes of fibroblast-like cells formed an open network and often ended in cell-cell contacts. The shape of the RER strands was similar to that observed in fibroblast-like cells of group 1, and lipid droplets were not often observed in these cells

either. Compared with group 1, there was increased lysosomal activity as judged from the presence of collagen containing vacuoles (CCV) and, occasionally, of irregularly-shaped electron dense lysosomal bodies (Fig. 7B). Myogenic characteristics were regularly seen in fibroblast-like cells of livers from rats containing low liver retinoid levels (Fig. 8).

Figure 6. Transmission electron micrographs of the liver of vitamin A sufficient rats after 8 weeks of CCl_4-treatment.
(a) Overview of a fibrous septum adjacent to parenchymal cells (P) and a sinusoid (S). Fibroblast-like cells (F), bile duct cells (B) and Kupffer cells (K) are in contact with the septum. Collagen fibrils are arranged into large, regularly-formed bundles and fill the extracellular space (arrows). (2656x).
(b) Fibroblast-like cell in fibrous septum. Dilated strands of rough endoplasmatic reticulum (arrow heads) and lysosomes (L) are present. Collagen bundles (C) are arranged in close proximity of the cell. (9130x).
(c) Fat-storing cell (F) in perisinusoidal space adjacent to parenchymal (P), Kupffer (K) and endothelial cell (E). (4399x). Inset: FSC show an activated appearance as judged by the presence of some short, dilated strands of rough endoplasmatic reticulum (arrow heads) and lysosomes (L). (13.515x).

Perisinusoidal FSC in regeneration nodules had few lipid droplets, revealed an activated appearance, and an increased condensation of basement membrane-like material in the subendothelial space (Fig. 7C).

Figure 7. Transmission electron micrographs of the liver of rats with a low vitamin A status after 8 weeks of CCl_4-treatment.

(a) Overview of a septum adjacent to parenchymal cells (P). In contrast to the liver with sufficient retinoid status, the extracellular matrix around the fibroblast-like cells (F) only contains a few focal collections of collagen fibrils (arrows). Most of the space is electron lucent or is filled with fuzzy material. B: Bile duct. (2656x).

(b) Fibroblast-like cells in fibrous septum. Dilated strands of rough endoplasmatic reticulum (arrow heads) are present. (7304x). Inset: A high lysosomal activity is observed in these cells, as is evident from the presence of collagen containing vacuoles. (22.440x).

(c) Activated fat-storing cell (FSC) in perisinusoidal space of a regeneration nodule, (P) parencymal cell; (E) endothelial cell. (9130x).

Figure 8. Transmission electron micrograph of a fibroblast-like cell (F) in the liver of a rat with a low vitamin A status after 8 weeks of CCl_4-treatment.

Myogenic characteristics, a distorted nucleus and subplasmalemmal densities (inset) were regularly observed in these cells. P: parenchymal cell; E: endothelial cell. (9130x). Inset: Note the presence of basement membrane-like depositions in close proximity to the fibroblast-like cell. (33.298x).

DISCUSSION

This experimental study shows that the vitamin A status of the liver has a modulating effect on the rate of induction of liver fibrosis by CCl_4. In vitamin A deficient rats, with low hepatic retinoid levels, liver fibrogenesis was accelerated and intensified as compared with rats with normal retinoid levels. This was manifested by higher hydroxyproline contents of the liver, lower liver weights and by accelerated development of cirrhosis. This increased tendency to develop fibrosis in response to CCl_4 treatment was also apparent in rats with a marginal vitamin A status.

Various liver diseases are associated with changes in retinoid metabolism and storage. Recent experimental studies have revealed that chronic ethanol administration results in a decrease of hepatic vitamin A content (12,13). Low retinoid levels have been observed in various types of human liver disease, particularly in alcohol- and drug-induced liver injury (14). These observations have led to speculation about whether a lowered retinoid level has an impact on liver fibrogenesis. This question was addressed in the present animal study, which employed the well-characterised model of CCl_4-induced fibrosis in rats. This has been shown to be useful for an understanding of the basic pathogenic mechanisms of liver fibrosis and cirrhosis (15).

In addition to the beneficial effects of vitamin A supplementation above normal requirements on experimental hepatic fibrosis (7,8), the results of the present study show that CCl_4-induced liver fibrogenesis is enhanced when liver retinoid levels are low or marginal. These observations strongly suggest that liver retinoids modulate hepatic fibrosis in rats.

The mechanism by which retinoids modulate hepatic fibrosis is not known. CCl_4 is hepatotoxic through the formation of free radicals by cytochrome P-450 in the endoplasmic reticulum of the liver (16). Liver fibrosis is induced following hepatocellular necrosis and inflammation. Previous work from our laboratory has shown that the hepatotoxicity of CCl_4 is enhanced when the retinoid status of the liver is elevated by oral administration of *excess* vitamin A (7). However, there was an inverse relationship between the severity of necrosis and the amount of collagen produced, i.e., rats with high liver retinoid levels had the most severe parenchymal cell necrosis but the least fibrosis in response to CCl_4. In the present study, there was no difference in the hepatotoxicity of CCl_4 observed between rats with normal or deficient retinoid status; collagen deposition was enhanced in livers with marginal or deficient retinoid levels. All these findings suggest that liver retinoids act directly on collagen synthesis or deposition in the liver. Therefore, we suggest that retinoids modulate collagen synthesis and deposition irrespective of the degree of hepatocellular necrosis induced by CCl_4.

Bearing in mind the relationship between retinoids and collagen deposition, a mechanism has been proposed for the increase in collagen synthesis resulting from CCl_4-

treatment (7). It has been shown that most of the retinoids of the liver are stored in the fat-storing cell (2,3,6). In addition, vitamin A content was shown to affect the phenotype of the fat-storing cell (7,17). Vitamin A storage in fat-storing cells appears to suppress the capacity of this cell type to synthesize collagen (17). These two main functions of the fat-storing cells, namely vitamin A storage and participation in fibrogenesis, appear to be inversely related. The reduction of retinoid levels in fat-storing cells and fibroblast-like cells by an enhanced secretion of retinol from the liver into the circulation during CCl_4-treatment, may stimulate the transformation of these cells to fibroblasts and, in this way, contribute to fibrogenesis of the liver. This may explain the accelerated progression to cirrhosis of livers with a low retinoid content.

Retinoic acid was added to the, otherwise retinoid deficient, diet in order to prevent accumulation of retinoids in the liver, without the development of an extrahepatic vitamin A deficiency syndrome. Retinoic acid supports the systemic functions of retinol, such as growth and epithelial cell differentiation (18), but cannot increase liver retinol and retinyl ester concentrations since it cannot be reduced to retinal or retinol. The effects of retinoic acid on phenotypic differentiation of the fat-storing cells by regulation of gene expression have been described (19,20). However, if any effect should exist, it does not explain the modulation of collagen synthesis in the present study because rats of all groups were fed the same amounts of retinoic acid.

The results of the present experimental study with the CCl_4-model indicate a further depletion of the hepatic vitamin A stores by CCl_4. These results are very similar to those observed during alcohol-induced liver disease, both in experimental animals and in man (12,21-26). Vitamin A depletion and lowered hepatic content of vitamin A may potentiate liver pathology in alcoholics, including the development of fibrosis and cirrhosis.

In summary, this experimental study significantly extends the notion that liver retinoids play an important role in hepatic fibrogenesis. Progression to cirrhosis is accelerated when liver vitamin A levels are low. The maintenance of sufficient vitamin A stores is probably an important expedient to minimalize the effects of exposure to fibrogenic agents. This may be of particular importance for those agents which exacerbate vitamin A deficiency of the liver, such as alcohol and CCl_4.

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C. BETA-CAROTENE (PROVITAMIN A) INHIBITS CCl_4-INDUCED HEPATIC INFLAMMATION AND FIBROSIS IN RATS

SUMMARY

Earlier data have shown that retinyl esters (vitamin A) strongly influence the effects of CCl_4 on the liver. The accumulation of collagen was inhibited, but an increased acute toxicity of CCl_4 was observed.

The present study was conducted to examine the effect of β-carotene (provitamin A) on the development of hepatic fibrosis induced by CCl_4 in rats. Oral administration of β-carotene during CCl_4-treatment resulted in a significantly lower increase in the hydroxyproline content of the liver and in less severe histopathological signs of liver fibrosis as compared with rats not treated with ß-carotene. The results of this study also showed that β-carotene administration could prevent the long-term loss of retinoids from the CCl_4-injured liver. No significant toxic side effects of β-carotene administration, such as found earlier with retinyl ester, were observed. The present experimental study suggests that β-carotene has a therapeutic potential in preventing liver fibrosis.

INTRODUCTION

Hepatic fibrosis, or excess deposition of collagen in the liver, is the most common lesion of clinically-relevant chronic liver diseases. In severe liver disease, fibrosis can progress to cirrhosis (1). Fibrosis and cirrhosis of the liver are associated with a high rate of morbidity and mortality. Therefore, early diagnosis as well as reversal and prevention of hepatic fibrosis in chronic liver diseases are important issues. At present, no satisfactory therapy for hepatic fibrosis exists. The pathogenesis of and the cells involved in the development of fibrosis of the liver are yet to be clarified.

Previously, we and others have reported that retinoids with vitamin A activity have a modulating effect on experimental CCl_4-induced hepatic fibrosis in rats. Senoo and Wake have shown that administration of retinyl palmitate inhibited hepatic fibrosis induced by either CCl_4 or pig serum (2). We also observed suppression of collagen accumulation, but there was also enhancement of acute toxicity, depending on the time of administration of vitamin A in relation to CCl_4-treatment (3). As a consequence, and also because large doses of retinol and retinyl esters can cause hypervitaminosis A (4), these retinoids are not likely to be useful for possible prevention or therapy of liver fibrosis.

Some carotenoids are a provitamin form of vitamin A which can be converted to

vitamin A in vivo (5,6). Cleavage of β-carotene into vitamin A and its derivates can occur in cells of the gut and therefore, β-carotene has vitamin A activity. However, in contrast to retinoids, β-carotene has been shown not to be toxic in vivo, even when taken in megadoses. Therefore, the present study was designed to examine the effects of β-carotene on CCl_4-induced hepatic fibrosis.

MATERIALS AND METHODS

Three-month-old female specific pathogen-free derived, inbred Brown Norway/ Billingham Rijswijk (BN/BiRij) rats, weighing 147 ± 9 g (mean ± SD, n = 34), were obtained from the Institute's colony (Rijswijk, The Netherlands). The rats were housed in groups of two or three animals in Makrolon type 3 cages and were maintained under "clean conventional conditions" (7). Water and a standard laboratory chow (Diet AM II, Hope Farms, Woerden, the Netherlands) were supplied ad libitum.

After a seven-day pre-experimental period, the rats were randomly divided into two groups of five rats and three groups of eight rats (Fig. 1). The mean body weight of the rats in the five groups (I-V) was similar. As shown in Fig. 1, rats of group I (= "CCl_4") received subcutaneous (s.c.) injections of 200 μl CCl_4 diluted in vegetable oil (vitamin A deficient sunflower oil; 1:1 v/v) per 100 g body weight twice weekly (Tuesday and Friday) for 4 weeks. Rats of group II (= "ß-carotene") were administered 12,500 IU β-carotene per 100 g body weight in 0.1 ml vegetable oil (vitamin A deficient sunflower oil) orally twice a week (Tuesday and Friday) for four weeks. Rats of groups III, IV and V received s.c. injections of CCl_4 as well as β-carotene orally twice a week for four weeks in the same concentrations as group I and II. As shown in Fig. 1, rats of group III (= "pretreatment") were pretreated with β-carotene. Two weeks after the last administration of β-carotene CCl_4 treatment was started. Rats of group IV (= "simultaneously") were administered β-carotene simultaneously with CCl_4 over the same time period. Rats of group V (= "post-treatment") were post-treated with β-carotene for four weeks, commencing two weeks after the last CCl_4 injection. When groups were not treated with CCl_4 or β-carotene, sunflower oil was given s.c or orally respectively.

Surgical liver biopsies

Surgical liver biopsies were taken to determine vitamin A and hydroxyproline content and to assess the morphology of the liver of the groups (Fig. 1). In the pre-experimental period (n = 5) and in week 11 (n = 4; all groups), rats were anaesthetized by halothane, and wedge biopsies of 50 to 75 mg of the median lobe were removed with an iris scissor (Stöpler, Utrecht, The Netherlands). A part of each liver biopsy was fixed in 4% buffered formaldehyde (pH 7.4) for light microscopy. The remainder was stored at -70°C for later

Figure 1. Experimental set up of the study.
Different groups of rats were treated with CCl_4 (I, n=5), with ß carotene (II, n=5), with ß carotene prior to CCl_4-treatment (III, n=8), with ß carotene and CCl_4 simultaneously (IV, n=8), or with ß carotene after CCl_4-treatment (V, n=8) (see material and methods).

analysis for vitamin A and hydroxyproline content. Surgical bleeding was staunched with dental foam (Willospon, Will Pharma B.V., Zwanenburg, The Netherlands) and the peritoneum and the abdominal wall were sutured with absorbable surgical suture (4-0 Dexon plus, Davis + Geck, Manati, U.S.A.). The skin was closed with wound clips (Clay Adams, Parsippany, U.S.A.) which were removed one week after surgery.

Liver preparation and histopathology

The rats were fasted overnight before sacrifice. At sacrifice, liver tissue was collected after perfusion with phosphate buffered saline (PBS, pH 7.4) through the portal vein for 5 min at 5 ml/min, followed by perfusion fixation. The median lobe was excluded from fixation by ligation and, after removal from the body, was weighed and immediately frozen in liquid nitrogen. The median lobe was stored at -70°C for later biochemical analysis. The remainder of the liver was fixed for light microscopy in neutral buffered formaldehyde. After fixation, the liver was removed from the body and weighed. Liver tissue was routinely embedded in paraplast and sectioned at 4 μm thickness. The sections were stained with haematoxylin-phloxin-saffron (HPS) and Sirius-red F3BA (8) for connective tissue and collagen.

The assessment of morphology was done by a pathologist working blind to the source of the specimens. Sections of liver biopsies and of post mortem liver tissue were analysed for the presence of inflammation and fibrosis. Parenchymal inflammation and liver fibrosis were graded from 0 to +++. Inflammation grade 0 reflected no inflammation, + the presence of scattered isolated inflammatory cells in the parenchyma, ++ focal collection of inflammatory cells, and +++ diffuse parenchymal inflammation; predominance of mononuclear or polymorphonuclear cell infiltration was noted. Fibrosis grade 0 reflected no fibrosis, + the presence of fibrotic tissue adjacent to the central veins (perivenular fibrosis), ++ bridging fibrotic septa between central veins and

between central and portal veins, and + + + cirrhosis. The scores for inflammation and fibrosis of the individual animals were cumulated and were expressed the mean ± S.E.M. (arbitrary units). The histological score for individual animals were statistically analysed using the Kruskal-Wallace test followed by Dunn's multiple comparisons according to Newman-Keuls (9).

Hydroxyproline determination

Hydroxyproline content, as an indicator for the amount collagen in liver, was determined using a modification of the method of Woessner (10), as described previously (11,12). Briefly, 25 to 30 mg or 300 to 500 mg of the frozen biopsies and median lobe of the liver, respectively, were lyophilized overnight, powdered and extracted twice with 2 or 20 ml diisopropylether, respectively, to remove vitamin A (which suppresses the colour development of the assay). Traces of diisopropylether were evaporated in a waterbath at 37°C. Hydrolysis was performed by addition of 5 ml of 6 N HCL per 300 mg "wet" tissue and incubation at 120°C for 16 h in screw-capped plastic tubes (Falcon 2070). After cooling, the hydrolysate was filtered through a 0.22 μm Millipore filter. Each tube and filter were rinsed with the same volume of 6 N HCL added to the first filtrate. The hydrolysates were stored at -20°C. A 100 μl sample of the hydrolysate was added to a mixture of 100 μl 6 N NaOH and 1.8 ml 0.3 M NaCl. One ml of 0.05 M chloramine T in citrate-acetate buffer (pH 6.0) in the presence of 1.32 M n-propanol was added to this mixture. The solution was mixed and incubated for 20 min at room temperature. The incubation was stopped by adding 1 ml perchloric acid/4-dimethylaminobenzoaldehyde solution in n-propanol. The colour was developed by a 15 min incubation at 60°C. After cooling, the absorbance was determined at 550 nm with an Ultraspec 4050 spectrophotometer (LKB, Bromma, Sweden). Hydroxyproline content was calculated on the basis of calibration curves using purified hydroxyproline as the standard.

Retinoid analysis

The liver content of retinol and the three main retinyl esters, i.e. retinyl palmitate, retinyl stearate and retinyl oleate, were determined using high performance liquid chromatography (HPLC) (13). The liver content of β-carotene was also determined using HPLC (14).

Biochemical analysis of blood

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyl transpeptidase (GGT) activities, as indicators of parenchymal liver cell damage, were determined in heparinized blood from the tail vein using the Reflotron system (Boehringer Mannheim, Germany) in the pre-experimental period and 11 and 18 weeks after the start of the experiment.

Statistics

Group means were evaluated for statistically significant-differences using two-tailed Student's t-test (significance level 5%, 1% and 0.1%).

RESULTS

Light microscopy

Histological evaluation of the surgical liver biopsies taken in the pre-experimental period showed normal liver morphology (not shown). At 11 weeks all liver biopsies taken from CCl_4-treated animals (group I, III, IV and V) showed steatosis, parenchymal cell necrosis and inflammation with polymorphonuclear and mononuclear cells in portal and central lobular areas. The severity of the lesions in both the β-carotene and non-β-carotene treated groups was similar. Parenchymal pericellular and dissecting fibrosis were present in the livers of the CCl_4-treated rats (group I, III-V). The pattern of fibrosis was the same in both β-carotene and non-β-carotene-treated groups but the degree of fibrosis was less severe in the β-carotene-treated groups (not shown).

Table 1. Body weight, liver weight and liver/body weight ratio of rats of group I-V (see materials and methods) at the time of sacrifice.

	I	II	III	IV	V
body weight (g)	192 ± 4	182 ± 4	192 ± 6	183 ± 2	183 ± 2
liver weight (g)	6.6 ± 0.1	5.9 ± 0.2*	5.6 ± 0.3*	5.8 ± 0.3*	5.8 ± 0.2*
ratio liver/body weight (%)	3.4 ± 0.1	3.2 ± 0.3	2.9 ± 0.3**	3.2 ± 0.4	3.2 ± 0.3

Values present the mean ± SEM.
* Significantly different from group I ($p < 0.05$).
** Significantly different from group I ($p < 0.01$).

Body and liver weight at sacrifice at 18 weeks are shown in Table 1. Moderate to pronounced inflammation (+-++) was present in the livers of the CCl_4-treated group (group I, Fig. 2). The average histopathological score of inflammation in the group treated with CCl_4 and β-carotene simultaneously (group IV) was lower when compared with group I (Fig. 2). The inflammation of portal tracts and parenchyma was usually of the mixed mononuclear - polymorpho-nuclear cell type with spotty and occasionally focal necrosis of the parenchyma. At 18 weeks, steatosis was not observed in the livers of either group (Fig. 3). Perivenular fibrosis was present in livers of all groups treated with CCl_4 (group I, III-V; Fig. 3). Bridging fibrotic septa between central veins and between

central and portal veins were also present, but the average histopathological scores of livers simultaneously (group IV) or post-treated with β-carotene (group V) were 37% and 31% lower, respectively, when compared with those of group I (Fig. 2). The lipid droplets content of fat-storing cells was less obvious in all groups treated with CCl_4, when compared with the ß carotene-treated group (not shown).

Figure 2. Histopathological scoring of inflammation and fibrosis in the liver after sacrifice. The rats treated with CCl_4 (I), with ß-carotene (II), with ß-carotene prior to CCl_4 treatment (III), with ß-carotene and CCl_4 simultaneously (IV) or with ß-carotene after CCl_4-treatment (V) (see material and methods).
Values represent the mean ± S.E.M.
a: Significantly different from group I, III and V, $p < 0.05$. b: Significantly different from group I and II, $p < 0.05$. c: Significantly different from group I, III-V, $p < 0.001$. d: Significantly different from group I and II, $p < 0.05$. e: Significantly different from group I and II, $p < 0.05$.

Biochemical observations

The normal control values of ALAT, ASAT and τGGT blood activities, as determined in the pre-experimental period, were 34 ± 3, 82 ± 6 IU/l and not detectable (< 5 IU/l), respectively (mean ± SEM; n = 5). At week 11, the activities in blood of CCl_4-treated rats were significantly increased (Table 2). ß-carotene given during the CCl_4-treatment did not significantly change the activities but, as indicated by lower ASAT and ALAT activities, there was a tendency towards reduced parenchymal liver cell damage. At the time of sacrifice, the activities in CCl_4-treated rats had decreased to control values, indicating that liver parenchymal damage has been recovered after CCl_4-withdrawal. ß-carotene given at the time of the CCl_4 injections or β-carotene administration after CCl_4 treatment (group II and V respectively) did not induce liver cell damage as indicated by low ALAT, ASAT and τGGT serum activities at 18 weeks.

Figure 3. Effect of β-carotene on CCl_4-induced liver injury.
Group I (a,b). Extensive fibrosis with subdivision of the parenchyma by thick fibrous septa (arrows) into small fields is seen after four weeks of CCl_4-treatment. Group II (c,d). No necrosis, steatosis or increased collagen deposition is observed in livers of rats treated with β-carotene alone. Group III (e,f). ß-carotene pretreatment had no significant effect on the degree of fibrosis induced by CCl_4. Group IV (g,h). Simultaneous treatment of rats with β-carotene and CCl_4 results in a lower degree of fibrosis when compared with CCl_4-treatment alone. Group V (i,k). Diffuse dissecting CCl_4-induced fibrosis is present in livers of rats post-treated with β-carotene but less extensive than in those of rats treated with CCl_4 alone.
a,c,e,g,i: HPS staining (44x); b,d,f,h,k: Sirius Red staining (28x)

a
b
c
d
e
f
g
h
i
k

Table 2. ASAT, ALAT and GGT activities in blood of rats treated with CCl_4 (group I), with β-carotene (group II), with β-carotene prior to CCl_4 treatment (group III), with β-carotene and CCl_4 simultaneously (group IV) and with β-carotene after CCl_4-treatment (group V) (see materials and methods).

Group	I	II	III	IV	V
11 weeks					
ALAT	530 ± 90	36 ± 2***	474 ± 143	392 ± 58*	538 ± 98
ASAT	856 ± 163	91 ± 7***	702 ± 99	591 ± 95*	877 ± 152
GGT	28 ± 5	ND	30 ± 3	28 ± 2	27 ± 4
18 weeks (sacrifice)					
ALAT	34 ± 1	44 ± 3	37 ± 2	32 ± 1	36 ± 1
ASAT	74 ± 3	95 ± 8	81 ± 5	71 ± 1	79 ± 3
GGT	ND	ND	ND	ND	ND

* Significantly different from CCl_4-treated groups I and V ($p < 0.05$).
*** Significantly different from CCL_4-treated groups I and V ($p < 0.001$).
Values are given as the mean ± SEM.
ND = not detectable (for GGT: < 5 IU/l).
ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; GGT = gamma glytamyl transpeptidase.

In the pre-experimental period, the livers contained 73 ± 9 μg retinoids per g of liver (mean ± SEM; n = 5). Retinoid levels (retinol and retinyl esters) of liver in the experimental period and at sacrifice are shown in Fig. 4. At 11 weeks, retinol and retinyl ester concentrations per gram of liver were significantly higher in the groups treated with β-carotene prior to, or during, CCl_4-treatment (group III and IV) as compared with groups treated with CCl_4 only (group I and V). The average concentrations in group III and IV were even higher when compared with untreated controls (group II), indicating the conversion of β-carotene to vitamin A during CCl_4-treatment. At sacrifice, the retinoid liver levels (μg per g liver) in rats treated with β-carotene (group II-V) were significantly higher than those in rats treated with CCl_4 only (Fig. 4). The results indicate that β-carotene administration prevents the long-term loss of retinoids from the CCl_4-injured liver. There was a difference in degree of β-carotene liver levels in group III, IV and V which was dependent on the time of administration of the β-carotene (Fig. 5); the lowest levels were found in livers of the group administered β-carotene prior to CCl_4-treatment (group III), the highest in livers of the group administered β-carotene after CCl_4-treatment (group V). Very high β-carotene liver levels were determined in group II as compared with those in liver of CCl_4-treated rats indicating that CCl_4-treatment reduces β-carotene levels of the liver.

Figure 4. Retinoid levels in liver at week 11 and after sacrifice (week 18).
The rats were treated with CCl_4 (I), with ß-carotene (II), with ß-carotene prior to CCl_4 treatment (III), with ß-carotene and CCl_4 simultaneously (IV) or with ß-carotene after CCl_4-treatment (V) at week 11 and 18. Values represent the mean ± S.E.M.
Week 11: a: Significantly different from group I and V (both groups were treated with CCl_4 only at this time point); $p < 0.05$.
Week 18: b: Significantly different from group I, III-V; $p < 0.001$. c:Significantly different from group I and II; $p < 0.001$.

Figure 5. ß-carotene levels in liver at sacrifice (week 18).
The rats were treated with CCl_4 (I), with ß-carotene (II), with ß-carotene prior to CCl_4 treatment (III), with ß-carotene and CCl_4 simultaneously (IV) or with ß-carotene after CCl_4-treatment (V). Values represent the mean ± S.E.M.
a: Significantly different from group I, III-V; $p < 0.001$. b: Significantly different from group I, $p < 0.05$ and from group II and V, $p < 0.001$. c: Significantly different from group I and V, $p < 0.05$ and from group II, $p < 0.001$. d: Significantly different from group IV, $p < 0.05$ and from group I-III, $p < 0.001$.

Normal control value of hydroxyproline content, as determined in the pre-experimental period, was 73 ± 9 μg per g liver (mean ± SEM; n = 5). Hydroxyproline content of liver in the experimental period and at sacrifice is shown in Fig. 6. At 11 weeks, the hydroxyproline concentration (μg per g liver) of the liver tissue of CCl_4-treated rats (group I, III-V) was significantly increased as compared with non CCl_4-treated rats (group II). As indicated by lower hydroxyproline concentrations, there was a tendency towards a reduction of collagen containing fibrotic tissue when β-carotene was given before or during CCl_4-treatment (group III and IV respectively). At the time of sacrifice, ß-carotene given before or after CCl_4-treatment (group III and V respectively) did not significantly change the hydroxyproline concentration in the liver when compared with the concentration of rats treated with CCl_4 only (group I). However, the concentration in liver of rats simultaneously treated with β-carotene and CCl_4 (group 4) was significantly lower as compared with rats treated with CCl_4 only (group 1). The

average concentration (μg per g liver) in the liver of rats in group IV was approximately 67 % of the amount determined in the livers of rats treated with CCl_4 only.

Figure 6. Hydroxyproline content in liver at week 11 and 18.
The rats were treated with CCl_4 (I), with ß-carotene (II), with ß-carotene prior to CCl_4 treatment (III), with ß-carotene and CCl_4 simultaneously (IV) and with ß-carotene after CCl_4-treatment (V). Values represent the mean ± S.E.M.
Week 11: a: Significantly different from group I and III-V; $p < 0.01$. b: Significantly different from group I, $p < 0.05$ and from group II, $p < 0.01$. c: Significantly different from group I and V (= CCl_4 treatment), $p < 0.05$ and from group II, $p < 0.01$.
Week 18: d: Significantly different from group I, III and V, $p < 0.01$ and from group IV, $p < 0.05$. e: Significantly different from group I and II, $p < 0.05$.

DISCUSSION

In a previous experiment we have examined the effects of vitamin A (retinyl palmitate) on CCl_4-induced liver inflammation and fibrosis and observed a dual effect of vitamin A on the induced liver disease (3). Briefly summarized, retinyl palmitate administered prior to, or during, CCl_4-treatment aggravated hepatotoxicity of CCl_4, and more than 50% of the rats died during CCl_4-treatment. However, when rats were given retinyl palmitate after CCl_4-treatment, none of the rats died and the dissecting fibrosis was less severe as compared with the fibrosis of rats which received CCl_4 treatment only. From these results we concluded that in the CCl_4 model, vitamin A administration seems to potentiate the hepatotoxicity of CCl_4 through metabolic interactions in parenchymal liver cells or by affecting the reactions of Kupffer cells, but we also concluded that a high level of vitamin A in fat-storing cells and fibroblastic cells in fibrotic septa probably suppresses the potential of these cells to contribute to liver fibrogenesis (3). These observations have led us to examine to what extent β-carotene can inhibit the hepatotoxic effects of CCl_4 and whether β-carotene can prevent the loss of retinoids from the CCl_4-injured liver which may inhibit fibrogenesis. For this purpose, β-carotene was administered prior to the initial injection of CCl_4, at the time of the CCl_4 injections or

after the last injection of CCl_4.

ß-carotene administered prior to CCl_4-treatment (group III) did not protect the rats against direct hepatotoxic effects of CCl_4 as indicated by high ASAT- and ALAT- blood activities and parenchymal cell damage observed in liver biopsies taken at one week after the last CCl_4 injection (week 11, Fig. 1), but the direct hepatotoxicity of CCl_4 was not augmented by β-carotene as it was by retinyl esters (3). At that time, vitamin A liver levels were significantly higher when compared with those in rats not treated with β-carotene (groups I, II and V). This may be the result of the conversion of β-carotene to vitamin A. Although the doses (expressed as IU) given in the present and previous study (3) were the same, retinoid liver levels in β-carotene-treated rats were approximately 45% of those in vitamin A treated rats. This must be due to the fact that only a part of the β-carotene is converted to vitamin A in vivo, dependent on the nutritional status (15).

Liver fibrosis was observed in the β-carotene-pretreated rats at the time of sacrifice but biochemical determination of collagen in the liver one week after the last CCl_4 injection revealed significantly lower levels than in the rats treated with CCl_4 only (Fig. 6). This indicates that β-carotene pretreatment cannot prevent liver fibrogenesis but can delay the induction of liver fibrosis in spite of parenchymal cell damage as reflected by high ALAT and ASAT blood levels. As shown in the previous study with retinyl palmitate (3), the elevated retinoid levels of the liver prior to CCl_4-treatment may be responsible for this effect. The delay of induction of liver fibrosis may be due to maintenance of high concentrations of retinoids in fat-storing cells which may postpone their activation to fibroblast-like cells.

When rats received β-carotene during the CCl_4-treatment the inhibition of fibrosis was still observed, but now the damage to parenchymal cells was also less. As mentioned before, the conversion of β-carotene to vitamin A and the uptake of the latter by fat-storing cells may contribute to the inhibition of liver fibrogenesis. Besides, it is conceivable that β-carotene may act as a scavenger of the very reactive chlorine because it is an excellent free radical trapping agent (16,17). Since hepatic fibrosis is probably stimulated by hepatocellular injury (18,19), this cytoprotective effect of β-carotene on the parenchymal liver cell could be an important mechanism of inhibition of hepatic fibrosis.

Histological and biochemical evaluation at the time of sacrifice showed that β-carotene post-treatment did not significantly reduce CCl_4-induced liver fibrosis. In contrast to retinoid post-treatment (3), only a minor decrease of liver fibrosis was found. Results showed that β-carotene administration supplied retinoids to the liver, but, at least in the set-up used (short periods), the increase of the retinoid levels may be too small to reduce collagen synthesis or deposits as observed by the administration of high doses of retinoids.

In summary, the results of this experimental study indicate that β-carotene gives no

apparent increase in systemic or hepatic toxicity of CCl_4 which may have promising implications for the prevention of drug-induced hepatic liver fibrosis in humans. The cytoprotective effects of β-carotene, particularly during drug-induced hepatic cell injury, may limit hepatocellular destruction and the accompanying fibrosis of the liver. The results of this experimental study have also shown that β-carotene treatment prevents the long-term loss of retinoids from the injured liver.

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CHAPTER 6

FAT-STORING CELLS AND MYOFIBROBLASTS ARE INVOLVED IN THE INITIAL PHASE OF CARBON TETRACHLORIDE-INDUCED HEPATIC FIBROSIS IN BN/BiRij RATS

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SUMMARY

This preliminary study on the appearance, distribution and kinetics of fibroblast-like cells (fat-storing cells, transitional cells, myofibroblasts and fibroblasts) after CCl_4-treatment was undertaken to further delineate the respective roles of these cell types in liver fibrogenesis. The different cell types were distinguished on the basis of their immunophenotypic pattern with a combination of marker antibodies and on the basis of ultrastructural characteristics.

Combined staining for alpha-smooth muscle actin (SMA) and desmin (DES) revealed perisinusoidal fat-storing cells (FSC) as d^+sma^- and myofibroblasts around the central veins of the normal rat liver as d^+sma^+. During the initial phase of CCl_4-induced hepatic fibrosis (week 1 and 2), the number of d^+sma^+ cells increased in the degenerating area around the central veins and d^+sma^+ cells appeared in the very thin fibrotic septa at week 2. Ultrastructural examination of the affected central areas showed the presence of myofibroblasts. These sma^+ cells proliferated, as shown by double staining for bromo-deoxy-uridine (BrdU) and SMA.

In degenerating parenchymal areas, d^+sma^- FSC were present. The FSC in the perisinusoidal space of areas which were not affected by CCl_4 intoxification, remained d^+sma^-. These immunostaining findings support the electron microscopical results, which show the presence of cells with the typical ultrastructural characteristics of FSC both in the degenerating areas and in the perisinusoidal space of unaffected areas.

After one week of CCl_4-treatment, enhanced deposition of procollagen type III was observed around the central veins. Enhanced deposition of collagen type IV was seen subendothelially along the sinusoids, notably in degenerating parenchymal areas where the septa were later formed. FSC appear to be the principal source of collagen type IV during fibrogenesis. These observations further support and specify the role of FSC in early fibrogenesis.

With the progression of the CCl_4-induced fibrosis, d^+sma^+ myofibroblasts remained localized in the fibrotic septa, but now along their outer edge. The majority of the cells in the septa was formed by d^-sma^- cells indicating a prominent role of fibroblasts in the septal formation. Septal fibroblasts are not only likely to produce matrix components, but also were shown to degrade collagen, as evidenced by the increased number of collagen-containing vacuoles during the course of fibrosis.

In conclusion, myofibroblasts and FSC appear to be the main cell types involved in the initial phase of liver fibrogenesis induced by CCl_4. Both myofibroblasts and FSC divide and transform. The results suggest that the d^-sma^- fibroblasts of well-developed fibrotic septa are derived from both d^+sma^+ myofibroblasts and d^+sma^- FSC. Septum formation is associated with the pattern of collagen type IV deposition.

INTRODUCTION

The regenerative capacity of the liver is so great that liver function can be restored within days, even after major acute damage induced by hepatotoxins. However, chronic exposure to hepatotoxins prevents the parenchyma from recovering from injury, and scar tissue (excess collagens, glycoproteins and proteoglycans) is formed which leads to remodelling of the liver architecture (fibrogenesis). The cellular processes and the nature of the cell type responsible for the formation of excess connective tissue components during fibrogenesis of the liver are not yet fully understood.

Various experimental models have been used which have contributed to the understanding of cellular and molecular mechanisms underlying excessive accumulation of fibrotic components in the liver. Animal models should closely approximate the pathogenesis of human liver fibrosis and cirrhosis. The carbon tetrachloride (CCl_4) model in rats is well documented. It resembles human liver fibrosis in many histological and biochemical aspects (1,4-6) and is, therefore, often used to study the formation of extracellular matrix components (5,7-9,59) and the proliferative potential of the various types of liver cells (10,11). In our laboratory, extensive fibrosis in rats is usually present after 4 weeks of treatment with CCl_4, and cirrhosis usually after 8 weeks (12).

Fibroblasts and fibroblast-like cells (myofibroblasts, fat-storing cells (FSC) and transitional cells (TC)) are associated with the production of collagen and other matrix proteins in liver. During fibrogenesis, the number of fibroblast-like cells increases, particularly in fibrotic septa, and the deposition of matrix proteins is enhanced. Since administration of excess retinoids to CCl_4-treated rats resulted not only in an increase in lipid droplet content of FSC, but also in the appearance of vitamin A containing lipid droplets in other fibroblast-like cells of the fibrotic liver (23), we have hypothesized that fibroblast-like cells of the liver are closely similar to FSC and may be derived from these cells.

The criteria which are used to distinguish the fibroblast-like cell types are reviewed in Table 1. In normal liver, FSC are localized perisinusoidally and are characterized by the presence of lipid droplets. Myofibroblasts and fibroblasts are localized around the central vein and in the portal tract. They both lack lipid droplets and have an extended RER. The immunophenotype of FSC is similar to that of myofibroblasts in expression for desmin (DES) and to that of endothelial cells in expression for collagen type IV. Myofibroblasts also express alpha-smooth muscle actin (SMA), whereas fibroblasts lack both DES and SMA expression. FSC and endothelial cells are the main cellular source of collagen IV. FSC and endothelial cells can be distinguished using rat endothelial cell antigen (RECA), which is a specific marker for endothelial cells.

As a result of liver cell damage, FSC may become activated and acquire some ultrastructural myogenic characteristics, with maintenance of DES immunoreactivity and

Table 1. Characteristics of the different non-parenchymal liver cells involved in CCl_4-induced fibrosis of rats.

	FSC	MF	TC	F	E
Morphology					
- lipid droplets	+/++	-	+	-	-
- extended RER	-/±	++	+	++	-
- remnants of basal lamina	-	+	-	-	±
- fil. dens.	-	+	+	-	-
- pinocytosis	±	++	+	-	++
- conv. nucleus	-	+	-/±	-	-
- intercell.junct.	-	±	±	+	+
- CCV	-	+	+	+	-
- lysosomes	±	+	+	+	+
Immunophenotype					
- type III collagen	+	?	+	+	-
- type IV collagen	-/+	?	+	-	-/+
- SMA	-	+	?	-	-
- desmin	+	+	+	-	-
- RECA	-	-	-	-	+
- laminin	-/+	?	?	-	-/+

References: **FSC:** 5,9,13,14,18,20,21,23,29,37,38,39,40,41,43,46,48,56,57,58,59,60;
TC: 5,9,13,14,18,20,21,23,38,39,40,41,43; **MF:** 28,40,42,44,45,52; **F:** 5,9,23; **E:** 32.

Criteria for identification

Ultrastructure

FSC: Cells containing lipid droplets; cells without basal lamina, filamentous densities and intercellular junctions; TC: Cells containing lipid droplets, extended RER, filamentous densities and possessing a moderate pinocytotic activity; no basal lamina; MF: Cells with remnants of basal lamina, rows of pinocytotic vesicles, filamentous densities, convoluted nucleus and extended RER; no lipid droplets; F: Cells with extended RER, a moderate pinocytotic activity, CCV, and intercellular junctions; cell without lipid droplets, basal lamina and filamentous densities; E: Cells lining sinusoids, lack a continuous basal membrane, and possess pinocytotic activity and tubular structures.

Immuno LM

FSC: The cells are desmin positive (d^+), sma^- and $RECA^-$. Some cells are positive for collagen type III and IV.
TC: collagen type III and IV positive; $RECA^-$.
MF: sma^+ and d^+; $RECA^-$.
F: collagen type III positive; type IV negative, sma^-, d^- and $RECA^-$;
E: Some cells are positive for collagen type IV; all cells are $RECA^+$; sma^- and d^-.

FSC = fat-storing cell; MF = myofibroblast; TC = transitional cell; F = fibroblasts; E = endothelial cell; CCV = collagen containing vescicles; SMA = α-smooth muscle actin; RECA = rat endothelial cell antigen.

lipid droplets. Because these activated cells combine lipid droplets and myogenic hallmarks they have been called TC (33). It has not been reported whether TC under these in-vivo conditions express SMA. An alternative origin of TC (eg myofibroblasts) cannot be excluded.

The cell types can be distinguished on the basis of their immunophenotypic pattern with a combination of marker antibodies, and also on the basis of ultrastructural characteristics. In the present study we analyze the involvement of FSC, myofibroblasts, TC, fibroblasts and endothelial cells in early fibrogenesis using both methods. The involvement of different cell types in the formation of fibrous septa is analyzed by the immuno-reactivity of cells for the above mentioned markers combined with bromo-deoxy-uridine (BrdU) incorporation as a marker for proliferation.

We have found that not only FSC participate in the formation of early septa but that a substantial part of the proliferating fibroblastic cell population consists of myofibroblasts and fibroblasts.

MATERIAL AND METHODS

Animals

Six-week-old female, specific pathogen-free, inbred BN/BiRij rats, weighing 170 ± 5 g (mean ± SEM, n = 16), were obtained from the Institute's Colony (Rijswijk, The Netherlands). The rats were maintained under "Clean Conventional Conditions" (34) and were housed in groups of two animals in Makrolon type 3 cages. Water and a commercial pelleted diet (AM2, Hope Farms, Woerden, The Netherlands) were supplied ad libitum. After a seven-day pre-experimental period, the rats were divided into four test and four control groups each of two rats. Mean body weight of the eight groups was similar. Rats of the test groups received subcutaneous injections of 200 μl CCl_4 diluted in vitamin A deficient vegetable oil (Reddy sunflower 0.1 l; 1:1 v/v) per 100 g body weight twice weekly for 1, 2, 3 or 4 weeks respectively. Rats of the control groups received vegetable oil only. For each time point two test and two control animals were examined in detail by immunocytochemical studies. In addition, the reproducibility of the time-course of induction of liver fibrosis by CCl_4 was tested by routine histopathological analysis (haematoxylin-phloxin-saphrane (HPS) and Sirius-Red (SR) stained sections) of the liver of two additional rats per time point, using the same treatment protocol.

Liver preparation

The rats were fasted overnight before sacrifice. One hour before sacrifice, bromo-deoxy-uridine (BrdU) was injected intraperitoneally (5 mg/100 g body weight). Under halothane anaesthesia the liver was perfused with phosphate-buffered saline (pH 7.4)

through the portal vein for 5 min at 5 ml/min, followed by perfusion fixation. The median and left lateral lobe were excluded from perfusion fixation by ligation. After they were removed from the body, the median lobe was immediately frozen in liquid nitrogen and was stored at -70°C for later use in making cryostat sections. The left lateral lobe was sliced and tissue slices were either fixed in an immersion of phosphate buffered-formalin (10% v/v) for 24 - 48 hours or fixed in acid-ethanol for 4 hours (4°C). The two remaining lobes were fixed by perfusion (5 ml/min for 8 min) by diluted Karnovsky (2% paraformalin and 2.5% glutaraldehyde).

For light microscopy the formalin and acid-ethanol fixed liver tissue slices were processed and embedded in paraplast according to routine procedures. Four μm thick paraffin sections were stained with HPS and SR to evaluate the liver architecture. For electron microscopy, Karnovsky fixed tissue was postfixed in 4% OsO_4 after being sliced into 100 μm thick sections with a vibratome. The osmificated tissues were dehydrated in graded concentrations of ethanol and embedded in Epon LX 112 (Ladd Research Industry Inc., Vermont, USA), according to routine procedures. Ultrathin sections were poststained with uranyl acetate and lead citrate and viewed with a Philips EM 410.

Immunohistochemistry

Cryostat sections and paraffin sections of acid-ethanol fixed tissue were used for immunostaining. Cryostat sections were mounted on gelatin-coated slides, air-dried, fixed in acetone and further processed for single or double labelling procedures. Paraffin sections were mounted on albumin-coated slides, cleared, and, if necessary, endogenous peroxidase activity was blocked in methanol with 0.3% H_2O_2. They were then washed in distilled water and processed for immunolabelling procedures similar to frozen sections. Antigens were indirectly labelled using fluorescent, peroxidase (PO) or alkaline phosphatase (AF) tagged secondary antisera or the alkaline phosphatase anti-alkaline phosphatase method (APAAP, 35). All antibodies were titrated for the different procedures for staining single- and double-staining to find the best working dilution. The primary antibodies of the first layer were incubated overnight at 4°C, whereas the primary antibodies of the second layer were incubated for one hour at room temperature (RT). The sections were incubated with secondary antibodies for 30 to 45 minutes at RT. To visualize collagen type IV and BrdU positive cells, sections were pretreated with pepsin (0.2%) for 30 min in 0.1N HCl at RT. Table 2 summarizes the specificity, dilution, use of frozen or paraffin sections, origin and abbreviations used of the primary and secondary mono- and polyclonal antibodies.

Double-staining of the mouse monoclonal anti-BrdU with the monoclonal antibodies directed to keratin 8 or SMA was done as follows: the primary mouse monoclonal antibody of the first layer was linked with RAM to APAAP, antigen sites on mouse Ig were destroyed and DNA denaturated in 2N HCl for 60 minutes at 37°C (36) successively

Table 2. Antibodies used in the present study

Specifity antibody (code)	Cell type	Dilution[1]	Detection2[2]	Ref3[3]	Origin[4]
primary antibodies					
monoclonal					
sma (1A4)	SMC, myo-fibroblasts	1/400 frozen	paraffin	(a)	Bio-maker
RECA-1	endothelial	1/1000	frozen	(c)	gift
polyclonal					
desmin	SMC, FSC	1/25	paraffin frozen	(d)	ED
collagen type IV		1/25	paraffin frozen	(e)	ED
procoll. type III (rat)		1/100	frozen	(f)	gift
secondary antibodies					
anti-BrdU		1/20		(b)	ED
RaAM-FITC		1/150			JL
GAR-TRITC		1/75			Nordic
RaAR-peroxidase		1/50			Nordic
SwAR-peroxidase		1/40			DAKO
GAM-peroxidase		1/50			DAKO
SwAR-AF		1/20			DAKO
RAM-AF		1/20			DAKO
mouse-APAAP		1/100			DAKO

1 Dilution stated is determined in an indirect immunoperoxidase assay.

2 Possibility of detection on paraffin or on frozen sections is stated.

3 (a) Skalli et al 1986 (52); (b) Schutte et al, 1989 (36); (c) Duijvenstijn et al 1992 (32); (d) Ramaekers et al 1983 (53); (e) Visser et al 1986 (54); (f) Schuppan et al 1986 (55).

4 Biomakor: DAKO:DAKOpatts, Glostrup, Denmark. ED.:Eurodiagnostics, Organon Teknika, Oss, The Netherlands. JL.:Jackson Laboratories, West Grove, USA.

Abbreviations used: BrdU: bromo-deoxy-uridin, EC: endothelial cells, FSC: fat-storing cells, sma: smooth muscle actin, SMC: smooth muscle cells, RECA: rat endothelial cell antigen, RaAM: rat-anti-mouse, GAR: goat-anti-rabbit, SwAR: pig-anti-rabbit, GAM: goat-anti-mouse, RAM: rabbit-anti-mouse, AF: alkaline phosphatase, APAAP: alkaline phosphatase-anti-alkaline phosphatase, TRITC: tetramethyl rhodamine isothiocyanate, fluorescin isothiocyanate: FITC.

followed by pepsin treatmen and detection of BrdU with anti-BrdU and RAM-peroxidase. The first antigen was visualised in the presence of levamisol (20 μM) with a Naphthol-AS-MX phosphate/Fast Red Violet alkaline phosphatase reaction to obtain a red colour deposit and the second antigen was visualised with a $CoCl_2$ enhanced DAB/H_2O_2 peroxidase reaction to obtain an black deposit. After the DAB reaction was stopped the sections were dehydrated in series of alcohol (70-100%) and mounted in malinol.

For the simultaneous detection of BrdU and desmin or collagen type IV; mouse monoclonal anti-BrdU was combined with rabbit polyclonal antisera. In the case of collagen type IV, sections were enzymatically-pretreated with pepsin. Sections were preincubated with normal rat serum and successively followed by incubations with rabbit antiserum, alkaline phosphatase tagged pig anti rabbit antiserum (SwAR-AF), 2N HCl (60 min at 37°C), pepsin treatment (if not yet done), mouse anti-BrdU and proxidase tagged goat anti mouse antiserum (GAM-PO). The activities for alkaline phosphatase and peroxidase were visualised as stated above.

For the simultaneous detection of desmin and α-sma, tetramethyl rhodamine isothiocyanate tagged goat anti rabbit (GAR-TRITC) and fluorescein isothiocyanate tagged rat anti mouse (RaAM-FITC) antisera were used as secondary antibodies. For fluorescent studies the cryosections were sealed in a drop of phosphate buffered glycerol with p-phenylenediamine (0.07%) (Sigma) as anti-fading reagent and viewed with a fluorescent microscope equipped with a Ploem-0-Pack.

Slides were thoroughly washed three times in phosphate-buffered saline between the different incubations. Positive and negative controls were always processed to assure the accuracy of the results. Negative controls included the omission of the primary antiserum or the replacement of the primary antibody by an irrelevant one (e.g. monoclonal antibody anti-keratin 10). The staining pattern of the various antigens obtained with the double staining procedures were carefully compared with, and found to be similar to, the staining patterns of the antigens involved, obtained after single immunostaining.

Histomorphometry

In light microscopy, BrdU positive cells were counted per 5 microscopic fields of 100 x magnification. BrdU positive cells were further analyzed phenotypically and 200 BrdU positive cells per liver were analyzed for their phenotype. In electron microscopy, all fibroblast-like cells were characterized and counted according to the criteria depicted in Table 1. Their localization around central veins, in septa and along the sinusoids was recorded (in 3 sections of 2 tissue blocks per animal).

RESULTS

Histology

Inflammation and fatty metamorphosis of the parenchyma were present in the central areas after one week of CCl_4-treatment. Liver cell necrosis, with ballooning of the parenchymal cells, was present. The inflammatory infiltrate was composed of mixed polymorphonuclear and mononuclear cell types. The deposition of collagen, visualized with Sirius-Red staining, was increased around the central veins (Fig. 1a). At one week, the number of fibroblast-like cells around the central veins was increased in the CCl_4-treated rats. After two weeks of CCl_4-treatment, very thin fibrotic septa were observed (Fig. 1b). At four weeks, bridging septa between central veins and between central and portal tracts were much thicker than at one week and enclosed areas of damaged hepatocytes (Fig. 1c).

Figure 1. Light microscopical analysis of collagen deposition after CCl_4-treatment. Sirius-red staining for collagen. Collagen deposits (arrow) are observed around central veins after one week of CCl_4-treatment (a, x 60); at two weeks of treatment, thin fibrotic septa connect central veins (arrow) (b, x 24); severe fibrosis, as indicated by moderately thick septa (arrow), is found after four weeks of treatment (c, x 24).
cv: central vein; s: septum.

Ultrastructure

In livers from control rats (n = 8), both myofibroblasts and fibroblasts were exclusively seen around the central veins (Table 3) and in the portal tracts. FSC were exclusively localized in the space of Disse. Transitional cells (TC) were not observed.

Table 3. Relative contribution of four fibroblast-like cells at one and two weeks after CCl_4-treatment as present around the central vein, portal tract, in septa and along sinusoid[a].

location and treatment		fat-storing cell	transitional cell	myofibro-blast	fibro-blast
central vein					
control	(n = 19)[b]	0	0	17	83
1 week CCl_4	(n = 42)	14	3	14	69
2 weeks CCl_4	(n = 35)	12	17	28	43
septa					
1 week CCl_4	(n = 9)	44	0	33	22
2 weeks CCl4	(n = 15)	13	7	13	67
perisinusoidal (space of Disse)					
control	(n = 52)	88	0	0	12
1 week CCl_4	(n = 17)	59	6	12	24
2 weeks CCl_4	(n = 18)	67	0	6	28

[a] The percentages of the four cell types are expressed.
[b] Number of cells counted in all sections (see material and methods).

Figure 2. Ultrastructural overview of the area around a central vein (CV) after one week of CCl_4-treatment. Note the presence of an injured hepatocytes (H), of fibroblasts (F), FSC and macrophages (mφ) (x 2,240). The inset shows a higher magnification of a transitional cell (TC) and fibroblast (F) surrounded by collagen fibrils (x 5,425). S: sinusoid.

After one week of CCl_4-treatment, FSC and an increased number of fibroblasts were observed in the perivenous areas (Fig. 2). As revealed by ultrastructural counting of FSC and fibroblast-like cells at one and two weeks of CCl_4-treatment (Table 3), the number and location of these types of liver cells were changed when compared with those in control livers. After one week of CCl_4-treatment FSC and TC appeared around the central veins (Table 3). The relative contribution of FSC to the the four fibroblast-like cell types localized in the space of Disse was reduced when compared with that in control livers (Table 3).

TC, myofibroblasts and fibroblasts were observed as a consequence of the CCl_4-treatment. Both myofibroblasts and FSC were located in the thin fibrotic septa and around central veins (Table 3). After two weeks of CCl_4-treatment the relative percentages of FSC and of MF in the septa were reduced. As a consequence, the relative percentage of fibroblasts was enhanced from 22% at week 1 to 67% at week 2. The ultrastructure of the different cell types mentioned above is illustrated in Figs. 3a-d.

After four weeks of CCl_4-treatment, myofibroblasts were observed mainly at the border of the fibrotic septa directly next to the parenchymal cells (not shown). Only a few FSC and TC were seen in these septa. Most of the cells were fibroblasts (Fig. 4A). This latter cell type possessed numerous intercellular junctions and electron-dense lysosomes of variable size (Fig. 4a and b).

An interesting ultrastructural finding was the presence of collagen-containing vacuoles (CCV) (Fig. 4a and b). We observed these vacuoles in myofibroblasts, TC and fibroblasts, always associated with lysosomes (Fig. 4b), but not in FSC although this cell type contained electron-dense lysosomes to a prominent extent (Fig. 4c). CCV were not found in fibroblast-like cells after one or two weeks of CCl_4-treatment but appeared after three weeks and were frequently observed after four weeks (in about 30% of the fibroblast-like cells counted in the ultra thin sections - between 40-50 cells counted per time point).

Immunophenotype

In normal liver, FSC are positive for DES and negative for SMA (abbreviated d^+sma^-, Table 4). Cells with this immunotype appeared to be evenly distributed in the liver parenchyma (confirmed by double immunofluorescence staining for DES and SMA, not shown). Vascular smooth muscle cells were generally, but not always, positive for DES and SMA ($d^{+/-}sma^{+/-}$). An intense deposition of collagen type IV was present around the central vein and in the portal tract around bile ducts and blood vessels. Weak deposition of collagen type IV was generally found along the sinusoids in the space of Disse. Immunolabelling for RECA revealed that the endothelial lining of the sinusoids, arteries and veins were intensely stained.

Figure 3a-d. Examples of the different fibroblast-like cells observed after CCl_4-treatment.

a. Fibroblast-like cell without lipid droplets in the space of Disse. This fibroblast has a similar ultrastructural appearance to FSC. The cytoplasm is filled with polyribosomes, some short extended strands RER and a large Golgi apparatus (G). Note the presence of a centriole. (x 8,500).

b. Transitional cell in the space of Disse. Note the presence of lipid droplets, the presence of myofilamentous densities (arrowheads), numerous pinocytotic vesicles (small arrows). The RER is inconspicuous. (x 11,390).

c. Myofibroblast near the central vein. Note the presence of myofilamentous densities (arrowheads), numerous pinocytotic vescicles (small arrows), moderately-developed RER and a distorted nucleus. Some lysosomes (L) are present. (x 7,200).

d. Fibroblast located between parenchymal cells (P). The fibroblast exhibited an extended RER (arrows). (x 8,500).

Figure 4a-c. Examples of collagen-containing vacuoles (CCV) in fibroblast-like cells.

a. The presence of CCV (arrowheads) is illustrated in a fibroblast. Note the presence of lysosomes (arrows) and a centriole (C). (4 weeks CCl_4, x 8,500)

b. Enlargement of a CCV (arrow) (x 26,400). Note the cell-cell contact (arrowhead).

c. The FSC possess large lipid droplets and some small electron dense lysosomes (arrows) but not CCV. Golgi apparatus and RER are inconspicuous (from the same preparation as "a" and "b", x 5,360).

Table 4. The staining pattern of the various fibroblast-like cells for desmin and smooth muscle actin in normal and CCl_4-damaged livers.

d^+,sma^-	Fat-storing cells in the perisinusoidal space of normal liver and in the perisinusoidal space of unaffected areas after CCl_4-treatment; fat-storing cells between CCl_4-damaged parenchymal cells, and in thin fibrotic septa and in the outer zone of thick fibrotic septa induced by CCl_4-treatment.
d^+,sma^+	Myofibroblasts around central veins and in portal tracts of normal liver; myofibroblasts in thin fibrotic septa and in the outer zone of thick fibrotic septa after CCl_4-treatment.
d^-,sma^-	Fibroblasts around central veins and in portal tracts of both normal and CCl_4-damaged livers; fibroblasts inside the thick fibrotic septa induced by CCl_4-treatment.

After one week of treatment with CCl_4 a striking similarity was observed between the pattern of collagen deposition, as visualized by the immunostaining for procollagen type III (Fig. 5a) and the pattern of sma^+ cells (Fig. 5b). The pattern of d^+ cells around

Figure 5a-i.
Immunoperoxidase staining for procollagen type III, alpha-smooth muscle actin, desmin, collagen type IV and RECA on sections of liver after one, two and four weeks of CCl_4-treatment.

a. Extensive staining of procollagen type III is observed around the central vein (CV) and radiating from it (arrows) after one week of CCl_4-treatment. Weak staining is also observed in the portal tract (PT, arrowheads) (x 16).

b. SMA positive cells (arrows) are observed only in the lining of central veins (CV) and portal tracts (PT) after one week of CCl_4-treatment (x 24).

c. DES positive cells (arrows) are observed along the central veins (CV, portal tracts (not shown) and also dispersed in the parenchyma after one week of CCl_4-treatment (x24).

d. SMA positive cells (arrows) are observed around central veins (CV) and in thin septa bridging central veins after two weeks of CCl_4-treatment (x 24).

e. DES positive cells (arrows) are observed in the parenchyma (P) and in the outer edge of the septa after four weeks of CCl_4-treatment. Only a few d^+ cells are observed around central veins (CV) (x 24).

f. SMA positive cells (arrows) are observed along the outer edges of the septa and not in the parenchyma (P) after four weeks of CCl_4-treatment (x 24).

g. Collagen type IV (arrows) is observed around portal tracts (PT) and central veins (CV) after one week of CCl_4-treatment but also in areas with damaged parenchymal cells (x 16).

h. RECA positive endothelial cells (arrows) are present along sinusoids, portal tracts (PT) and central veins (CV) after one week of CCl_4-treatment (x 24).

i. RECA positive endothelial cells (arrows) are observed along sinusoids and in septa after four weeks of CCl_4-treatment (x 24).

central veins was similar to the pattern of sma $^+$ cells but in addition d^+ cells were found in the parenchyma (Fig. 5c). During the next two weeks, d^+ cells in the perisinusoidal space were still identified as sma$^-$ FSC, also when they were located between fat loden hepatocytes in regenerative areas after CCl_4-treatment (Table 4). However, d^+ cells that had accumulated around the central vein or in septa were also sma$^+$ (Fig. 5d). After four weeks of CCl_4-induced fibrosis, myofibroblasts (d^+ and sma$^+$) were only localized along the outer edge of the thick septa, while the fibroblast-like cells inside the fibrous septa had no immunoreactivity for DES and SMA (d^-sma$^-$) (Fig. 5e and f). Enhanced deposition of collagen IV was seen subendothelially along the sinusoids, after one week of CCl_4-treatment (Fig. 5g). The deposition was concentrated in areas between major vessels where later the septa were formed. Collagen type IV positive cells (c.f. Fig. 7c) had an elongated appearance. Parenchymal cells were never positive for collagen IV staining. Endothelial cells were stained for RECA at one and four weeks of CCl_4-treatment (Fig. 5h and i).

Table 5. The number of BrdU positive cells and the percentage of cells positive for both BrdU and smooth muscle actin or desmin or RECA or collagen type IV.

	number of BrdU$^+$/5MF	% of total BrdU			
		SMA	desmin	RECA	collagen IV
control	< 1[a]	nd	nd	nd	nd
1 week	128 ± 9	6,10[b]	10,13	1,1	6,8
2 weeks	182 ± 25	11,14	12,16	nd,7	5,14
4 weeks	578 ± 71	10,15	10,18	2,7	8,9

[a] The mean value ± standard deviation of BrdU$^+$ cells counted per 5 microscopic fields (MF) of 100 x magnification (n = 8).

[b] The percentages are based on 200 BrdU$^+$ cells counted per liver and are given for the individual cases.

nd not done.

Proliferation

The number of BrdU$^+$ cells and the relative distribution of the BrdU$^+$ cells among the various cell types are shown in Table 5. In control livers less than one BrdU$^+$ nucleus was seen per five microscopic fields (100 x magnification). The number of BrdU$^+$ cells increased from 128 at one week of CCl_4-treatment to 578 at four weeks. Based on the morphological features about 75% of the BrdU$^+$ cells were identified as parenchymal cells or bile epithelial cells at all timepoints of CCl_4-treatment (Figs. 6a-c). A small number of BrdU$^+$ cells seen in the portal area during the first two weeks of treatment

Figure 6a-c. Immunoperoxidase staining for BrdU on sections of liver after one week of CCl_4-treatment.

a. BrdU positive staining is observed in parenchymal cells (barred arrow), bile epithelial cells (arrowhead) and other cells (arrows) (x 60).

b. BrdU positive staining is observed in areas of bile duct cell proliferation (arrows) and in endothelial (barred arrow) and fibroblast-like cells (arrowhead) in damaged parenchymal areas (x 96).

c. BrdU positive staining is present in biliary epithelial cells (arrow), parenchymal cells (barred arrow) and in fibroblast-like cells (f) and other non-parenchymal cells (arrowhead) (x 96).

were interpreted as proliferating macrophages on the basis of nuclear morphology (not shown). About 20% of the $BrdU^+$ cells exhibited a fibroblastic appearance and these cells were found around the central veins, within the portal areas and between parenchymal cells (Figs. 6a-c).

Some examples of double staining for BrdU and different cell markers (SMA, 7a; DES, 7b; collagen IV, 7c; RECA, 7d) are shown in Fig. 7. The percentage of $BrdU^+sma^+$ or $BrdU^+d^+$ cells varied from about 8% and 12%, respectively, in the first week and from about 12% and 14%, respectively, after 4 weeks (Table 5). In fibrotic liver about 40% of the $BrdU^+d^+$ positive cells were located in parenchymal areas (Fig. 7b) and since these cells were sma^-, they were interpreted as proliferating FSC or TC. The rest of the $BrdU^+d^+$ (= 60%) was present in septa. $BrdU^+d^-$ fibroblastic cells were also seen in the septa. Some of the endothelial cells (= RECA-1 positive) were BrdU labelled (Fig. 7d), indicating the formation of blood vessels in the septa (Table 5). At one week, $BrdU^+$-collagen IV^+ cells were observed in the parenchyma and, later, in or close to septa (Fig. 7c). Their percentage varied from 5 to 14% (Table 5).

DISCUSSION

The present study on the appearance and distribution of fibroblast-like cells (FSC, TC, myofibroblasts and fibroblasts) during the first weeks of CCl_4-treatment was undertaken

Figure 7a-d. Double immunostaining for BrdU and sma (a), BrdU and desmin (b), BrdU and collagen type IV (c), and BrdU and RECA (d) in livers from rats treated for one week (a) and four weeks (bd-d) with CCl_4.

a. Note the presence of sma^+BrdU^+fibroblast-like cells (arrow) around the central vein (CV) (x 100).
b. d^+BrdU^+cells are present in areas with injured parenchymal cells (x 160).
c. Collagen type IV^+ $BrdU^+$ cell is present in the parenchyma (x 160).
d. $RECA^+$ $BrdU^+$ endothelial cells (arrows) are onbserved in the injured parenchyma (x 160).

to further delineate the respective roles of these cell types in liver fibrogenesis. Although the number of rats examined in this preliminary study was small, the time course of induction of liver fibrosis with this schedule of CCl_4-treatment is reproducible in this strain, and the nature of the early cellular and extracellular changes appeared to be consistent. The observations were largely in line with those from other time-dependent studies on CCl_4-induced liver fibrosis, while some new findings based on the detection of a more complete set of cellular marker antigens may shed a new light on existing notions on the early pathogenesis of CCl_4-induced liver fibrosis.

In normal liver, identification of all cell types is feasible on the basis of their

localization and ultrastructural appearance, and by immunohistochemical techniques detecting marker molecules such as SMA, DES and vitamin A. However, in fibrotic and cirrhotic liver the liver architecture is altered, other cell types appear (e.g. TC) and cell number, localization and phenotype of normal cells are changed (FSC, MF, bile duct cells, parenchymal cells). Therefore, identification of cell types is difficult and requires a more extensive analysis. In the present study, we used a combination of (immuno) LM and EM techniques to distinguish cell types during liver fibrogenesis.

The nature of the cell type responsible for the formation of connective tissue components during fibrogenesis of the liver has long been elusive, even in the intensively studied model of CCl_4-induced liver fibrosis. FSC, myofibroblasts as well as fibroblasts have been reported to participate in the process of fibrogenesis (20,37-43). Most recent reports have strengthened the view that FSC rather than other fibroblast-like cells are primarily responsible for the increased deposition of extracellular matrix proteins in fibrotic liver (41,43). These conclusions were based on DES as an unequivotal marker for FSC. The validity of DES as a marker for FSC is, however, limited because myofibroblasts contain DES as well (28), and the interpretation of the role of each of these cell types in the process of fibrogenesis may also depend on the DES antibody used. Moreover, the role of fibroblasts, which lack DES, could not be taken into consideration. Vitamin A autofluorescence, another marker for FSC, is also not reliable, because the presence of vitamin A in FSC depends on the vitamin A status, which can be greatly reduced during liver fibrogenesis (37). Administration of high doses of vitamin A increases the vitamin A stores, not only in FSC, but also in fibroblasts located in the interstitium around the central veins and in the portal tracts of the normal and fibrotic liver (23, also supported by other unpublished EM observations from our laboratory).

Labelling for SMA gives additional information about the role of myofibroblasts (28,40,44,45). We confirmed the cell-specific labelling pattern of SMA in normal liver: combined staining for DES and SMA revealed FSC as d^+sma^- and myofibroblasts around the central veins of the normal liver as d^+sma^+.

During the initial phase of CCl_4-induced hepatic fibrosis (week 1 and 2), the number of d^+sma^+ cells increased in the degenerating area around the central veins and appeared in the very thin fibrotic septa. Ultrastructural examination of the affected central areas showed the presence of myofibroblasts, indicating that myofibroblasts in fibrotic sections remain sma^+. These cells proliferate, as shown by double staining for BrdU and sma, which may explain the increased number of myofibroblasts in the injured areas. In these areas, d^+sma^- cells were also present, indicating the involvement of FSC in the initial phase of fibrogenesis. These findings are in accord with reports on the involvement of d^+ cells in fibrogenesis (40,43,46). The FSC in the perisinusoidal areas which were not affected by CCl_4 intoxification, remained d^+. These immunofluorescence findings support the ultrastructural results showing the appearance of FSC in the

degenerating areas and the existence of FSC in the perisinusoidal areas. Therefore, we conclude that both myofibroblasts and FSC are the main cell types involved in the initial phase of liver fibrogenesis induced by CCl_4.

After just one week of CCl_4-treatment, enhanced extracellular deposition of collagen type IV was seen subendothelially along the sinusoids, notably in parenchymal areas between major vessels where later the septa were formed. Northern hybridization analysis of isolated and purified parenchymal and non-parenchymal cells of normal liver showed that collagen type IV mRNA was expressed mainly in FSC and endothelial cells of the sinusoid (31). Since endothelial cells, in contrast to FSC, contain only small amounts of type IV mRNA (31), and, furthermore, EC were not numerous in the degenerating areas around central veins as indicated by RECA-1 immunolabelling in the present study, FSC may be the principal source of this protein during fibrogenesis. These observations further support and specify the role of FSC in early fibrogenesis, although it cannot be fully excluded that myofibroblasts might also produce type IV collagen during fibrogenesis (Table 1).

With the progression of the CCl_4-induced fibrosis d^+sma^+ myofibroblasts remain localized in the fibrotic septa, but are now found along their outer edge. The biological role of d^+sma^+ myofibroblasts is speculative. They are potentially contractile, in view of their myogenic filaments, and may, therefore, be involved in the process of scar contraction. The majority of the cells in the septa are formed by d^-sma^- cells indicating a prominent role of fibroblasts in septal formation. The fibroblasts, which contain little or no SMA or DES, are in a synthetic phase. Detection of procollagen I, III and IV mRNA with the in situ hybridisation technique revealed that the fibroblasts produce all of these proteins (9,11). Septal fibroblasts not only produce matrix components, but they also degrade them, as evidenced by the increased number of collagen-containing vacuoles (CCV) during the course of fibrosis. These CCV have been described in tissues in which active remodelling occurs (47). The fate of the phagocytosed collagen fibrils is lysosomal digestion (47). The collagenous network of fibrous septa is loosely organized after 4 weeks of treatment, which suggests that the excess matrix production may be compensated by phagocytosis and degradation by fibroblasts.

FSC, TC, myofibroblasts and fibroblasts of the rat liver are fibroblast-like cells which are related. The condition of the liver parenchyma determines the phenotypic expression of the fibroblast-like cells. In normal liver FSC are perisinusoidal cells that participate in the storage of vitamin A and in the maintenance of the sinusoidal structure of the liver lobules. After an assault on the liver, the FSC of injured areas become activated and are transformed to TC that appear to have phenotypic and biochemical features of fibroblasts and myofibroblastic cells (37,39,40,48,49). In a previous study, we found that newly-administered vitamin A can also be stored in fibroblasts of the fibrotic septa induced by CCl_4, which provides new evidence for the idea that these cells are derived

from FSC (23). Vitamin A changed not only their phenotype expression towards FSC or TC but also reduced collagen deposition in the fibrotic liver indicating a reduced or increased capacity of these cells to synthesize or catabolize collagen, respectively. The relation of FSC, myofibroblasts and fibroblasts is also confirmed by in vitro studies. FSC, with d^+sma^- immunoreactivity, isolated from normal rat liver, transform spontaneously into myofibroblastic cells in culture, expressing both DES and SMA (50). Moreover, cultured skin fibroblasts express SMA (51) indicating that the acquisition of myogenic characteristics is stimuli-dependent. Standard culture conditions (which include fetal calfs serum) stimulate cell division and transformation including synthesis of extracellular matrix proteins. These processes can be further modified by various growth factors some of which are likely to be produced after tissue damage and during fibrosis. The results of the present study are in line with the view of phenotypic modulation of fibroblast-like cells. TC appeared both along the sinusoids and in the scars, indicating the transformation of FSC, as did myofibroblasts. Furthermore, proliferation of d^+sma^- FSC or d^+sma^+ myofibroblasts was observed in the degenerating area around the central veins and in septa between adjacent central veins of the liver resulting in an increase of d^-sma^- fibroblasts during the course of hepatic fibrosis indicating phenotypic modulation of liver FSC, TC and myofibroblasts into fibroblasts.

In conclusion, the results of the present preliminary study suggest that the d^-sma^- fibroblasts of well-developed fibrotic septa are derived from both d^+sma^+ myofibroblasts and from d^+sma^- FSC. During the early formation of septa, both myofibroblasts and FSC divide and transform. Locally increased collagen type IV deposition may be one of the earliest phenomena in the formation of septa.

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CHAPTER 7

THE EFFECT OF LONG-TERM ETHANOL ADMINISTRATION AND CALORIE RESTRICTION ON SURVIVAL AND AGE-RELATED PATHOPHYSIOLOGICAL CHANGES IN RATS

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SUMMARY

Life-long restriction of calorie intake increases life-span of mammals and delays or prevents age-associated disease processes. Chronic alcoholism is a common cause of both liver disease and chronic pancreatitis particularly in conditions of specific physiological or pathological vulnerability, such as advanged age. The present study was designed to investigate the consequences of chronic ethanol administration and life-long calorie restriction on age-related pathophysiological changes and survival of rats. Age-related changes in physico-chemical properties of tissue collagen were investigated because of their potential to serve as biological markers of ageing.

For the survival study, 3-month-old male BN/BiRij rats were randomly divided over two groups, one ad libitum (AL) and one on 65% calorie restriction (CR) by "every-other-day-feeding". Rats of both groups received drinking water containing 25% (v/v) ethanol. There was no significant difference in ethanol intake per unit body weight or in blood ethanol concentrations between the two groups. The 50% survival of AL-fed ethanol rats was only 13 months, while the CR group was reached at an age of about 27 months.

None of the ethanol treated rats in either group displayed light microscopic abnormalities of the liver, such as steatosis, hepatitis or fibrosis. Severe inflammation of the intestines, pituitary tumours and kidney disease (pyelitis) were observed frequently in the ethanol/AL rats, whereas ethanol/CR rats did not show these abnormalities, even though they had been exposed to ethanol for a longer period. Testicular atrophy, keratinization of tail skin and thinning of the fur were observed in all ethanol fed animals.

There was a strong age-related increase in tail tendon breaking time and a decrease in tissue collagen solubility in AL control rats. The age-related increase in tail tendon breaking time was retarded by calorie restriction. Ethanol-fed CR rats had comparable tail tendon breaking times as age-matched non-ethanol fed CR rats.

INTRODUCTION

Life-long restriction of calorie intake increases the life-span of animals and delays or prevents age-associated disease processes (1). Such studies were mainly done in animals kept under "optimal" laboratory conditions and whether these experimental findings can be extrapolated to humans still remains unclear. The human population is, in comparison with inbred laboratory animals, genetically diverse and exposed to a very complex and variable environment and life-style. One important life-style factor present in our society

is chronic ethanol consumption. Chronic alcoholism is a common cause of both liver disease and chronic pancreatitis particularly in conditions of physiological or pathological vulnerability, such as advanged age (11). The present study was designed to investigate the consequences of chronic ethanol administration and life-long calorie restriction on age-related pathophysiological changes and survival of rats. Age-related changes in physico-chemical properties of tissue collagen were investigated because of their potential to serve as biological markers of ageing (2,3).

MATERIALS AND METHODS

Animals

Male BN/BiRij rats (n = 20) were kept on normal lab chow (AMII, Hope Farms, Woerden, The Netherlands) ad libitum up to an age of 3 months. At that time, the rats were randomly divided over two groups, one of which received AMII-diet ad libitum (AL), the other was kept on restricted AMII-diet (CR) by "every-other-day-feeding" (20 h per 48 h) (4), resulting in about 65% of the AL food intake. All rats of both the AL and CR group received drinking water containing 25% (v/v) ethanol either as whisky/water (n = 5 per group) or as whisky/beer (n = 5 per group).

Non ethanol-fed ad libitum or calorie restricted, control BN/BiRij rats of various age groups were obtained from the aging cohorts of the Institute. The normal survival characteristics and background age-related pathology of male and female AL rats of this strain have been described in detail earlier (5).

At the time of sacrifice, livers were perfused and partly fixed for light and electron microscopy and partly frozen for biochemical analysis, as described in detail by Bosma et al. (6). In addition, plasma and skin samples were obtained. Intact tendons were removed from the tail. Histopathological analyses were done after routine post mortem examination, essentially as described earlier (5,6).

Analyses

Alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma-glutamyl transpeptidase (GGT), glucose, triglycerides, cholesterol and urea were determined in plasma by routine procedures, as described earlier (7). Ethanol concentrations in blood were determined spectrophotometrically on samples collected at 6.00 am and 6.00 pm. Hydroxyproline, an amino acid unique to collagen, was determined according to Woessner, as described earlier (7).

Collagen extractibility was determined in skin, tail tendon and liver by homogenization of lyophilized tissue in 0.1% (v/v) acetic acid and overnight rehydration at 4°C, as described by Snowden et al. (8). After centrifugation, this procedure was

sometimes followed by a second solubilization step of the 84,000 g.min pellet in the same solvent at 80°C for 1 h. Solubilization of skin collagen required more stringent conditions than used for tail tendon. Hydroxyproline content of original tissues and extracts was analyzed to determine collagen solubility.

Tail tendon breaking time was determined according to Harrison and Archer (3), using a standardized weight of 2.00 g and 9 M urea at 45°C.

RESULTS

Food and ethanol intake and body weight

The food intake was 14 g per day in the AL rats, and about 65% of the AL intake in the CR rats. The body weights of AL rats were about 30% higher than of CR rats (Table 1).

Table 1. Body weight of ethanol-treated ad libitum and calorie restricted rats.

Age (month)	Ad libitum Whisky	Whisky/beer	Calorie restricted Whisky	Whisky/beer
12	350 ± 10 (5)	370 ± 9 (5)	281 ± 6 (5)	265 ± 5 (5)
15	372 ± 22 (2)	366 ± 21 (3)	290 ± 6 (4)	270 ± 5 (3)
23	381 ± 10 (2)	365 ± 6 (2)	290 ± 8 (4)	268 ± 18 (2)
27	-	-	290 ± 8 (4)	215 ± 57 (2)
33	-	-	281 ± 6 (2)	235 (1)

At the start of the experiment, when the rats were 3 months old, body weight was 259 ± 5 (20).
Values are given as means ± SEM (n).

After an habituation period of 13 weeks, the ethanol-intake was stabilized at 120 ± 7 (S.D.) and 124 ± 10 g ethanol/kg body weight/week, for AL- and CR-rats, respectively. There was no significant difference in ethanol-intake per unit body weight between the two groups. The ethanol-intake remained constant thereafter. Blood ethanol concentrations at 6.00 a.m. and at 6.00 p.m. were 1.09 ± 0.40 (S.D.) and 0.59 ± 0.40 g/l,

respectively, for AL rats, and 0.83 ± 0.52 and 0.59 ± 0.40 g/l, respectively, for CR rats, with no significant differences between the groups.

Survival

A clear difference in survival was noted between the two dietary groups (Fig. 1). In both groups, the first deaths occured at an age of 11 months. The 50% survival time of ad libitum-fed (AL) ethanol rats was only 13 months, while the 50% survival time of the calorie restricted (CR) group was 27 months (Fig. 1). At 33 months of age, when 4 of the 10 CR rats were still alive, the experiment was terminated.

Figure 1. Survival of ad libitum fed (AL) and calorie restricted (CR) male BN/BiRij rats, which were maintained on drinking water containing 25% ethanol.
Arrows indicate the age at which the last remaining rats of each group were sacrificed.

Histopathological observations

Histological examination was done both on rats that died spontaneously and on sacrificed animals. For the ethanol/AL group, tissues were obtained between ages 11 and 24 months (n = 10). For the ethanol/CR group, the ages ranged from 11 to 33 months (n = 10). None of the ethanol treated rats in either group displayed light microscopic abnormalities of the liver, such as steatosis, hepatitis or fibrosis. This is a remarkable finding, that indicates that, at least in this rat strain, even life-long high ethanol intake is not hepatotoxic if it is combined with a nutritionally adequate diet.

Extra-hepatic ethanol-related pathology was observed in the ethanol/AL rats: Severe inflammation of the intestines was present in 60%, pituitary tumors in 40%, and, kidney disease (pyelitis) in 40% of the rats. The ethanol/CR rats did not show these abnormalities, even though they had been exposed to ethanol much longer. Testicular atrophy, keratinization of tail skin and thinning of the fur were observed in all ethanol-fed animals and these changes were interpreted as signs of premature aging, since they also occured in non-ethanol fed control AL rats, although at later ages.

Biochemical parameters in plasma

The plasma values of alanine aminotranspeptidase (ALAT), aspartate aminotrans-

peptidase (ASAT), and, gamma glutamyl transpeptidase (GGT) were the same as in normal control animals, in both AL and CR ethanol-treated rats. Only some animals that were killed when moribund displayed slightly elevated values of ALAT and ASAT (not shown). These data underline the absence of ethanol-induced primary liver cell damage in these rats.

Solubility and mechanical properties of tissue collagen during aging

Tail tendon. Extraction with acetic acid at 80°C for 1 h was very effective for tail tendon and always solubilized > 95% of tendon collagen, also in old control rats (not shown). Figure 2 shows that, under milder conditions (overnight extraction in 0.1% (v/v) acetic acid at 4°C), the percentage solubility of tail tendon collagen decreased from almost 100% at 3 months of age to below 10% at 38 months.

Figure 2. Solubility of tail tendon collagen of female BN/BiRij rats of various ages.
Tail tendon collagen was extracted with 0.1% acetic acid at 4°C overnight, as described in Materials and Methods. Bars represent S.D. (n = 4-7)

The tail tendon breaking time showed a reverse age-related pattern to that of collagen solubility. There was a dramatic age-related increase in breaking time in AL control rats from about 4 min at 3 months of age to over 24 hr at 42 months of age. This increase was linear on a semi-logarithmic scale (Fig. 3).

Figure 3. Effects of ageing on tail tendon breaking time of ad libitum fed female BN/BiRij rats.
Bars represent S.D. (n = 4-7).

Skin and hepatic collagen. The solubility of skin collagen also decreased with age, both in 0.1% acetic acid and in PBS, from 70% in young (6-month-old) rats, to 25% in 24- and 36-month-old rats (not shown). For liver collagen, a gradual decrease in extractibility was noted up to 21 months of age, but, surprisingly, the extractibility was significantly increased at 35 months of age when compared to the values at 12 and 21 months of age (Fig. 4).

Figure 4. Solubility of hepatic collagen of male BN/BiRij rats of various ages. Hepatic collagen was extracted with 0.1% acetic acid at 4°C and 80°C, sequentially, as described in Materials and Methods. Values are means ± S.D. (n = 4, except for 35-month-old (n = 3)). The 6- and 35-month values are significantly different (*: $p < 0.05$) from both the 12- and 21-month values.

Effects of long-term calorie restriction and ethanol administration. The solubility of skin collagen was 86 ± 3% (n = 3) in female calorie restricted rats at 29 months of age, and significantly lower in age-matched ad libitum rats (66 ± 3%; $p < 0.05$). Calorie restriction inhibited the age-related increase in tail tendon breaking time (Table 2). Surprisingly, the 32-month-old ethanol-treated CR rats displayed a breaking time that was not only much shorter than that of their age-matched AL controls, but also shorter than that of untreated 29-month-old CR rats (Table 2).

Table 2. Effect of calorie restriction and ethanol consumption on tail tendon breaking time.

Treatment	Age (month)	Gender	Breaking time (min)
Ad libitum	29-30	F	394 ± 231 (8)
Calorie restriction	29	F	78 ± 11 (8)*
Ad libitum	32-34	M	302 ± 90 (6)
Ethanol/restriction	32	M	34 ± 5 (5)*

Values are mean ± S.D. (n).
* Significantly different from age-matched ad libitum control ($p < 0.05$).

DISCUSSION

These long-term experimental studies on the combined effects of calorie restriction and ethanol consumption in rats display clear differences in survival and pathophysiology as a result of the dietary treatment. In ad libitum (AL) fed rats, ethanol administration resulted in a reduction of the average survival age from 31 months in AL non-ethanol treated (control) rats to about 13 months. Apart from the normal pathology of senescent rats (5), ethanol-fed AL rats often displayed disorders of the gastro-intestinal tract and kidneys, notably inflammation, which could be due either to the direct damaging effect of ethanol on the tissues or indirectly on the immune system (6,9).

Although some rats died at a very early age in both the AL and CR group, calorie restricted ethanol-fed rats had a much longer 50% survival age of about 27 months and they did not develop pathology that could be specifically related, directly or indirectly, to the effects of ethanol. In addition, strain- and age-related tumors (5) were not found in food restricted animals. These differences between the AL and CR group were not likely to be the result of differences in ethanol intake or metabolism, since plasma ethanol levels were similar in both groups.

From these long-term experiments with ethanol in combination with calorie restricted food intake, some extrapolation to the human situation might be justified. Because of evident metabolic differences between rats and humans, extrapolation of these experimental results should be done with great caution. However, the basic concept of nutrition being a powerful modulator in the pathogenesis of age- and of alcohol-related disease might also be true in humans. In view of the high, still increasing, chronic alcohol consumption in the Western society, these experimental data may strengthen the possible significance of calorie restriction as an achievable regimen for reduction of age-related pathology and, thereby, of life prolongation. More specifically, the results highlight that both the hepatic and extrahepatic long-term effects of ethanol may be depend on nutritional factors (6,9,10). Therefore, further definition of the biochemical mechanisms, which are active in the interaction between ethanol and nutrition, is warranted.

One of the important problems in defining aging at an experimental level is the lack of dependable biomarkers of aging. Physicochemical properties of tissue collagen have been proposed as such a marker (2,3). In the present study, we have confirmed that solubility of skin and tendon collagen decreases with age and that tail tendon breaking time increases. As expected, calorie restriction significantly slowed down both processes. Surprisingly, no consistent age-related decrease in solubility was found for liver collagen. Ethanol-fed CR rats had comparable tail tendon breaking times as age-matched non-ethanol fed CR rats. Since the average survival of non ethanol-fed CR rats is much longer (> 38 months, unpublished results), these results suggest that the use of this parameter as a biomarker of aging should be done with caution.

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CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

8.1 INTRODUCTION

The pathogenesis of alcohol-related liver disease is complex and still requires considerable investigation particularly in advanced stages of liver disease under continued alcohol abuse (1). Only 17-30% of heavy drinkers develop alcoholic liver disease (2,3). This suggests that even daily consumption of large quantities of ethanol may not be hepatotoxic per se. Extrahepatic processes and etiologic factors including nutrition, immune function and genetic background are likely to contribute to the development of alcoholic liver disease.

To improve our knowledge of human alcoholic liver disease animal models have been employed. Since decades, investigators have tried to develop suitable experimental models of alcoholic liver disease, of which fibrosis is the hallmark. To date, no animal model fully reproduces the disease stages observed in man. The criteria, for an ideal model include the following (4):

- duplication of the sequence of the pathophysiological and morphological alterations observed in human alcoholic liver disease;
- gradual progression of the pathologic changes;
- reversibility of early stages and irreversibility of the stages of advanced fibrosis and cirrhosis.

In addition, high reproducibility and low initial mortality are required to make the model effective in experimental studies and fulfil criteria necessary for statistical significance of results. Despite the inherent limitations of animal models (see chapter 1.7), in the studies described in this thesis various experimental models have been used which contribute to the understanding of cellular and biochemical mechanisms underlying excessive accumulation of extracellular matrix components in the liver (see chapter 1.3), in relation to the effects of ethanol but also to carbon tetrachloride.

In chapter 2 and 3, we investigated the effects of long-term ethanol administration

in rats on the liver. The effects were studied in relation to malnutrition and nutritional factors, particularly vitamin A and protein-fat composition of the diets. The rats were administered ethanol in a liquid diet for 12 to 16 months.

In chapter 4, 5 and 6, short-term studies in rats with carbon tetrachloride (CCl_4) were conducted to study the pathogenic and cellular mechanisms in liver fibrosis and cirrhosis. The aims of these studies were to determine whether: **a)** alcohol consumption, which had little effect on liver in normal rats even after a very long period of alcohol administration, would affect fibrogenesis in liver of rats previously treated with CCl_4 (chapter 4); **b)** vitamin A status and hepatic retinoid content have an impact on the induction of liver fibrosis and cirrhosis by CCl_4. Chapter 5A reports the effects of excess retinoid administration on liver fibrogenesis and recovery and chapter 5B reports on CCl_4-induced fibrogenesis in rats which livers were deficient for retinoids; **c)** β carotene had a therapeutic potential on developing liver fibosis (chapter 5C); and **d)** fibroblastic cells which are involved in liver fibrogenesis were related to fat-storing cells or to myofibroblasts. Chapter 6 gives an analysis of the morphogenesis of the fibroblastic cells during liver fibrogenesis.

In chapter 7, the consequences of chronic alcohol consumption and life-long calorie restriction on survival and ageing were investigated.

8.2 LONG-TERM EXPERIMENTS WITH ALCOHOL IN RATS

8.2.1 Combined effects of ethanol and vitamin A

In 1963 a liquid diet pair-feeding technique of alcohol administration was introduced by Lieber and DeCarli (4). Ethanol was administered to rats as part of a nutritionally defined liquid diet. This diet contained 36 % ethanol, 35% fat, 18% protein and 11% carbohydrate as percentages of the total calorie intake. In the control liquid diet ethanol was substituted by isocaloric carbohydrate. With this technique ethanol intake was achieved in rats resulting in high blood alcohol levels.

In 1983, Lieber and co-workers reported for this rat model that liver fibrosis could be elicited within 9 months when substantial amounts of vitamin A were added to the alcohol containing liquid diet (5). This rat model of alcohol administration does not reflect the human situation for several reasons. Rats were administered the alcohol-containing diet to which five times the normal amount of vitamin A was added, resulting in a high vitamin A content of the liver. In humans, vitamin A content of the liver is known to be lowered in alcoholic liver disease (6). Another major drawback of these experiments was the very young age of the rats (weanlings) at the start of the treatment, which is not the usual situation in man. Also, the type of liver fibrosis induced was different. Ethanol-related fibrosis in human liver starts by pericentral and centro-central

septal localization of fibrotic areas, but the rats fed the high vitamin A-ethanol diet for 9 months developed, besides variable degrees of pericentral fibrosis, periportal fibrosis (5).

We applied the same treatment protocol as Lieber and coworkers and, in addition, a protocol with lower vitamin A concentrations. We used the BN and WAG rat strains instead of the Sprague-Dawley rat strain of Liebers group. Young adult rats were used because earlier results had shown that administration of alcohol to weanlings affected their maturation and growth and resulted in a poor general condition and health status.

Our results show that long-term treatment of WAG and BN rats with ethanol in the retinoid-supplemented Lieber-DeCarli diet does not result in liver abnormalities that are characteristic of human alcoholic liver disease. The slight inflammatory response in the liver parenchyma was non-specific and had no characteristics of alcoholic hepatitis as observed in humans. A gradual progression of pathological changes towards liver fibrosis was not observed. Therefore, the findings of Leo and Lieber cannot be confirmed (5), and the role of vitamin A as a major factor in the pathogenesis of alcoholic liver fibrosis should be reconsidered. As has been observed in experiments with other fibrotic agents like CCl_4, it is possible that an high retinoid content of the liver of ethanol-treated rats may suppress the induction of liver fibrosis. The failure to reproduce the morphologic features characteristic of human alcoholic liver disease and the lack of reproducibility of liver fibrosis, as found by Liebers group, limits the value of the ethanol-high vitamin A containing Lieber-DeCarli rat diet in the study of alcoholic liver disease.

8.2.2 Combined effects of ethanol and malnutrition

The importance of alcohol and nutrition in alcoholic liver disease has long been the subject of intense debate between those who considered malnutrition to be the primary injurious factor and those who thought that alcohol was the primary cause of the liver disease, independent of nutrition (7,8). Data generated from human studies of 20-30 years ago led to a general consensus that alcohol is a direct hepatic toxin and that diets calculated to be nutritionally adequate do not protect against this effect (8). While this conclusion tends to minimize the role of nutrition in alcoholic liver disease, the discovery that, for instance, high fat intake in combination with high alcohol consumption may be associated with a histological picture indistinguishable from alcoholic liver disease requires a reassessment of the association between alcohol and nutrition (7). Recently, in a prospective study of chronic alcoholics, it was reported that nutrition had a beneficial effect on alcohol-induced liver damage (9).

The role of nutrition in alcohol liver disease was examined in a long-term experiment with rats which were fed a fat- and carbohydrate-rich, calorie sufficient, but otherwise poly-deficient liquid diet, which was qualitatively more or less comparable to the food intake among chronic alcoholics (10). The diet contained 37% of the total calories as

ethanol, namely whisky. For each ad libitum ethanol-fed rat, there was a paired carbohydrate-fed control.

The study was an extension of earlier experiments in rats on the effects of chronic alcohol administration on the liver under different nutritional conditions. We found that life-long alcohol administration to rats maintained on normal lab chow has severe pathological effects on various organs but not on the liver. In another study, we found a fibrogenetic effect of alcohol when its administration was combined with carbon tetrachloride (CCl_4), a known potent hepatotoxic and fibrogenetic agent. Furthermore, we got good evidence that nutritional factors, notably vitamin A, modulate liver fibrosis induced by CCl_4.

The poly-deficient liquid diet was very rich in fat which provided 38% of total calorie intake. Intake of high dietary fat and high alcohol has been associated, both in experimental animals (11) and in humans (12), with liver damage additional to the production of steatosis. Recent studies by French and co-workers have addressed the issue of the amount of dietary fat as a critical factor for alcohol-related liver damage in experiments with dietary manipulations (13) comparable to those applied in our study. However, their model is artificial in that it involves administration of both ethanol and food through permanent intra-gastric catheters. In addition, unscheduled mortality is high, both as a result of surgical complications, and of ethanol intoxication due to very high blood ethanol levels (11,13,14,15). In their studies ethanol administration leads to centro-central septal fibrosis, but only a few of the rats were still alive after 6 months of treatment (15).

Three months after the start of the experiment, steatosis was observed in all livers of both alcohol-treated and control rats. Steatosis as such may be the effect of high fat intake, protein-calorie malnutrition or of the combination of both, but not the effect of alcohol alone. The results of the study in chapter 7 support this idea because chronic ethanol administration combined with normal, protein and calorie sufficient, lab chow did not induce steatosis, or any other liver abnormalities.

The addition of alcohol as whisky to the fat-rich protein-deficient diet resulted in an enhancement of liver fibrogenesis as reflected by a higher hydroxyproline content and the histological findings of the liver. The critical values for blood alcohol levels and the amounts of dietary fat required for induction of liver fibrosis in rats have not yet been defined, but the amount and type of fat in alcohol-containing diets and the high sustained blood alcohol levels are likely to be important in determining the severity of the alcoholic liver damage, including fibrosis (14). Rats in the French model of intragastric alcohol infusion developed severe steatosis and necrosis, often with fibrosis of the liver, on a diet containing 25% of total calories as fat and on blood alcohol levels averaging 353 mg/dl (13). In our study, the rats developed liver fibrosis on a dietary fat content of 38% of total calories and on blood alcohol levels ranging from 50-126 mg/dl.

The vitamin A content of our diet was low, and so were the hepatic retinoid levels of rats of both groups. From studies with the CCl_4-model, we got good evidence that the maintenance of sufficient vitamin A stores is an important expedient to minimalize the effects of exposure to fibrogenic agents. Low hepatic vitamin A levels, which can be the result of low dietary intake but may also be due to interference with vitamin A metabolism by agents such as ethanol (16), may be a risk factor for the development of liver fibrosis. The high lipid droplet content of both fat storing cells and septal fibroblasts reported by French and Tsukamoto (13) stongly suggest that their diet contained much more retinoid than the diet we administered. On the one hand, this may have lowered the incidence of fibrosis unintentionally, since excess vitamin A, although possibly augmenting hepatocellular damage, is known to suppress hepatic fibrogenesis (chapter 5A). On the other hand, their conclusions on the role of proliferation of fat storing cells in septa formation is questionable, because we have found that septal fibroblasts can acquire vitamin A containing lipid droplets after administration of high doses of the vitamin.

In summary, these experimental findings confirm the previous experimental and clinical indications that protein- malnutrition with high fat intake contributes to the fibrogenesis of the liver associated with chronic high alcohol consumption. In this respect, malnutrition could be the triggering factor necessary for the induction of so-called alcoholic liver disease.

8.2.3 Effects of alcohol and nutrition on ageing

In Western society, alcoholic beverages are considered part of the basic food supply. A large daily intake of alcohol results in various toxic effects which may reduce human lifespan as indicated by results of our experimental studies in rats. In contrast to alcohol consumption, adequate nutrition may increase human lifespan as suggested by experimental studies with animals kept on long-term restriction of calorie intake (17). This nutritional regiment results in a delay or even prevention of age-associated disease processes (17). The consequences of a combination of chronic ethanol administration and long-term calorie restriction on lifespan of rats were studied. In this study, age-related changes in physico-chemical properties of tissue collagen were also investigated, because of their potential to serve as biological markers of aging (18,19).

This study displayed clear differences in survival and pathophysiology as a result of dietary treatment. In ad libitum (AL) fed rats, ethanol administration resulted in a reduction of the average survival age to about 13 months, as compared with 31 months in AL non-ethanol-treated (control) rats. Apart from the normal pathology of senescent rats, ethanol-fed AL rats often displayed disorders of the gastro-intestinal tract and kidneys, notably inflammation, which could be due either to the direct damaging effect of alcohol on the tissues or by an indirect effect on the immune system (chapter 2, 1).

Calorie restricted (CR) ethanol-fed rats had a much longer 50% survival age than AL ethanol-fed rats (27 and 13 months, respectively). CR rats did not develop pathology that could be specifically related, directly or indirectly, to the effects of ethanol, even though they had been exposed to ethanol for a longer time. These differences between the AL and CR groups were probably not the result of differences in ethanol intake or metabolism, since plasma ethanol levels were similar in both groups. These results were confirmed in a recently finished follow-up experiment.

From these long-term experiments with alcohol in combination with calorie restricted food intake, some extrapolation to the human situation may be justified. Because of evident metabolic differences between rats and humans, extrapolation of these experimental results should be done with great caution. However, the basic concept of nutrition being a powerful modulator in the pathogenesis of age- and of alcohol-related disease might also be true in humans. In view of the high, still increasing, chronic alcohol consumption in Western society, these experimental data may strengthen the possible significance of calorie restriction as an achievable regimen for reduction of age-related pathology and, thereby, of life prolongation. More specifically, the results highlight that both the hepatic and extrahepatic long-term effects of alcohol very strongly depend on nutritional factors. Therefore, further definition of the biochemical mechanisms, which are active in the interaction between alcohol and nutrition, is warranted.

8.3 SHORT-TERM EXPERIMENTS WITH CARBON TETRACHLORIDE IN RATS

We have used the carbon tetrachloride (CCl_4) model in various studies to examine the deposition of extracellular matrix and efficacy of retinoids and β carotene as antifibrogenic agents. CCl_4-induced fibrogenesis requires repeated episodes of focal centrilobular necrosis in which fibrosis results from collapse of the existing reticulin framework and de novo collagen synthesis. Regeneration of the parenchyma evolves from surviving liver cells forming nodules within connective tissue septa. The model has a good reproducibility at our laboratory.

8.3.1 Effects of ethanol on CCl_4-induced liver fibrosis

Alcohol-induced histological lesions in a short-term experimental rat model were compared with those characteristic of human alcoholic liver disease. In the rat model used, pre-treatment with CCl_4 for six weeks was employed to sensitize the liver for the effects of alcohol and to shorten the time of induction of alcoholic liver disease. Subsequent maintenance on drinking water containing up to 10 percent alcohol for seven weeks potentiated liver fibrosis, as compared with non-alcohol-treated rats. Rats post-treated with normal drinking water displayed a partial recovery of the liver fibrosis.

Administration of alcohol to CCl_4-treated rats resulted not only in inhibition of the recovery of the liver, but also in further progression of collagen deposition in fibrous septa. However, increased liver fibrosis was the only histological evidence of alcoholic liver disease observed; the histological pictures of steatosis and alcoholic hepatitis comprising damaged parenchymal cells with Mallory body formation and inflammatory response with mainly polymorphonuclear cells were not observed within the seven-week period of alcohol administration. This experimental study shows that alcohol consumption can be an important determinant of the course of liver fibrosis in rats. This experimental approach may be useful for studying the effects of alcohol consumption on liver damage and fibrosis induced by other agents such as methotrexate and other hepatotoxins.

8.3.2 Effects of (pro)vitamin A on CCl_4-induced liver damage

A. Hypervitaminosis A

The relation between vitamin A and liver fibrosis is complex. Both hyper- and hypovitaminosis A status of the liver have been associated with alcoholic liver injury and fibrosis both in man and in experimental studies (6,20,21). Experimental studies showed that vitamin A may act synergistically with ethanol in the generation of liver injury and fibrosis (5), while it was also reported to suppress the induction of fibrosis by CCl_4 and heterologous serum (22).

The effect of vitamin A (retinoids) administration on CCl_4-induced liver damage and fibrosis was examined. In these experiments, a dual effect of vitamin A on the hepatotoxicity of CCl_4 was observed, which was related to the time of administration. Vitamin A enhanced the hepatotoxicity of CCl_4 when administered prior to or during CCl_4 treatment. Significant mortality was found, in contrast to CCl_4-treatment alone. The mechanism of enhanced toxicity of vitamin A in combination with CCl_4 is not known. It was proposed by Lieber (23) that vitamin A is metabolized by liver microsomes, particularly when cytochrome p-450 activity is stimulated by a hepatotoxic agent such as CCl_4. The vitamin A metabolites produced might play a role in the increased hepatotoxicity of CCl_4. The enhanced toxicity of vitamin A in the liver during CCl_4-treatment might, alternatively, be related to Kupffer cell activation. Hypervitaminosis A is associated with activation of Kupffer cells, which may lead to increased secretion of mediators which can induce parenchymal cell damage (24). The high doses of vitamin A were not hepatotoxic when they were administered after CCl_4-treatment, and resulted in enhanced removal of CCl_4-induced fibrotic septa. This may be due to effects of vitamin A on the metabolism of both fat-storing cells and fibroblast-like cells in the fibrotic liver. The results showed that newly administered vitamin A was stored in both cell types, which may reduce their collagen synthesis, as was seen in cultured fat-storing cells loaded with vitamin A before isolation (25).

In conclusion, a high vitamin A status of the liver suppresses experimental liver fibrogenesis. This may be explained by high concentrations of retinoids in fat-storing-cells and fibroblast-like cells, which suppresses the potential of these cells to contribute to fibrogenesis of the liver. However, vitamin A may potentiate the hepatotoxicity of agents such as CCl_4 through metabolic interactions in parenchymal cells or by affecting the response of Kupffer cells.

B. Hypovitaminosis A

Experimental studies by other workers have established that chronic ethanol administration results in a decrease of hepatic vitamin A content (6,21). Low retinoid levels have also been observed in various states of human liver disease, particularly in alcohol- and drug-induced liver injury (21). These observations and the results described above have led to the question whether a low retinoid level has an impact on liver fibrogenesis. The effects of lowered vitamin A status of the liver on CCl_4-induced liver fibrosis were reported.

The study showed that also a vitamin A status below the normal range has an important impact on liver fibrogenesis. The vitamin A status of the liver appears to have a strong modulating effect on the rate of CCl_4-induced liver fibrosis. Although there was no difference in the degree of hepatocellular damage induced by CCl_4 between rats with normal or deficient retinoid status, liver fibrogenesis was accelerated and intensified in rats with low hepatic retinoid levels when compared to rats with normal retinoid levels. This was manifest by higher hydroxyproline contents of the liver, by lower liver weights and by accelerated development of parenchymal regeneration nodules. These observations suggest that retinoids modulate the increase in collagen synthesis and deposition which occurs in response to hepatocellular necrosis.

Together with the results of the hypervitaminosis A study, these findings suggest that liver retinoids act directly upon collagen synthesis or deposition in the liver. If the retinoid content in the liver is high or is increased by treating rats with retinoids prior to repeated injections of CCl_4, hepatic fibrosis is inhibited. The results in chapter 5B show that CCl_4-induced liver fibrogenesis is enhanced when liver retinoid levels are low or reduced. Although the cellular mechanisms responsible for the increased collagen synthesis resulting from CCl_4-treatment are not fully understood, the results of the studies with low and high vitamin A status indicate that a reduction of retinoid levels is a key step in the activation of fat-storing cells and that maintenance of high concentrations of retinoids in fat-storing cells can reduce such activation and, in this way, fibrogenesis.

In summary, this experimental study significantly extends the notion that liver retinoids play an important role in hepatic fibrogenesis. Progression to cirrhosis is accelerated when liver vitamin A levels are low. Furthermore, the risk of lowered hepatic retinoid stores is increased as is indicated by a further depletion of the hepatic vitamin

A stores during CCl_4-treatment. The maintenance of sufficient vitamin A stores is probably an important expedient to minimize the effects of exposure to fibrogenic agents. This may be particularly important for those agents, such as alcohol, which exacerbate vitamin A deficiency of the liver.

C. Beta-carotene

As a sequel to the studies with retinoids on CCl_4-induced hepatic fibrosis, an experiment was designed to examine the possible effects of the carotenoid β-carotene, which can be converted to vitamin A in vivo. In contrast to retinoids, this naturally occurring form of vitamin A has shown to be relatively non toxic, even in high doses. Furthermore, it has also been observed that β-carotene is an excellent free radical trapping agent and, in fact, may be a very efficient scavenger for radicals caused by bioactivation of hepatotoxic drugs.

Oral administration of β-carotene during CCl_4-treatment reduced parenchymal cell damage and liver fibrosis. These observations suggest that co-administration of β-carotene suppresses both the hepatic parenchymal necrosis and the formation of liver fibrosis induced by CCl_4. It is conceivable that the reduction in hepatocellular necrosis is the result of scavenging of the very reactive chlorine radical by β-carotene in the liver, which could reduce hepatic parenchymal cell necrosis. Since hepatic fibrosis is probably stimulated by hepatocellular injury (26,27), this cytoprotective effect of β-carotene on the parenchymal cell could be an important mechanism in the suppression of hepatic fibrosis.

Results of this experimental study also showed that β-carotene treatment prevents the long-term loss of retinoids from the injured liver. This is likely to be due to the conversion of β-carotene to vitamin A. Since hepatic vitamin A probably prevents or reduces collagen formation by liver fat-storing cells and fibroblast-like cells, administration of the provitamin A β-carotene may have a promising therapeutic potential against liver fibrosis.

8.4 NON-PARENCHYMAL LIVER CELLS AND FIBROSIS

The cellular processes and the nature of the cell type responsible for the formation of excess connective tissue components during fibrogenesis of the liver are still partly known. The preliminary study on the appearance, distribution and kinetics of fibroblast-like cells (fat-storing cells, transitional cells, myofibroblasts and fibroblasts) after CCl_4 treatment was undertaken to further define the respective roles of these cell types in liver fibrogenesis. The different cell types were distinquished on the basis of their immunophenotype, using a combination of marker antibodies, but also on the basis of ultrastructural characterization.

We confirmed the cell-specific labeling pattern of alpha-smooth muscle actin (SMA) and desmin (DES) in normal liver: combined staining for DES and SMA characterized fat-storing cells as des^+sma^- and myofibroblasts around the central veins of the normal liver as des^+sma^+. During the initial phase of CCl_4-induced hepatic fibrosis, the number of des^+sma^+ cells increased in the degenerating area around the central veins and these cells emerged in the very thin fibrotic septa at week 2. Ultrastructural examination of the affected central areas showed the presence of myofibroblasts. These cells proliferate, as was shown by double staining for bromo-deoxy-uridine (BrdU) and SMA, which explains the increased number of myofibroblasts in the injured areas.

In the degenerating areas around central veins, des^+sma^- cells were also present, which indicates the involvement of fat-storing cells in the initial phase of fibrogenesis. The fat-storing cells in the perisinusoidal areas which were not affected by CCl_4 intoxification, also remained des^+sma^-. These immunofluorescence findings support the ultrastructural results showing the appearance of fat-storing cells in the degenerating parenchymal areas and the presence of fat-storing cells in the perisinusoidal areas. Therefore, we conclude that both myofibroblasts and fat-storing cells are the main cell types involved in the initial phase of liver fibrogenesis induced by CCl_4.

After one week of CCl_4-treatment, enhanced depositions of collagen type IV were seen subendothelially along the sinusoids, notably in parenchymal areas between central veins where later the septa were formed. Northern hybridization analysis of isolated and purified parenchymal and non-parenchymal cells of normal liver showed that collagen type IV mRNA was expressed mainly in FSC and endothelial cells of the sinusoid (28). Since endothelial cells, in contrast to fat-storing cells, contain only small amounts of type IV mRNA (28), and, furthermore, endothelial cells were not numerous in the degenerating areas around central veins, as indicated by RECA-1 immunolabeling, fat-storing cells may be the principel source of this protein during fibrogenesis.

With the progression of the CCl_4-induced fibrosis, des^+sma^+ myofibroblasts remain localized in the fibrotic septa, but are now found only along their outer edge. The majority of the cells in the septa is formed by des^-sma^- cells indicating a prominent role of fibroblasts in the septal formation. Septal fibroblasts do not only produce matrix components, but they also degrade it, as indicated by the increased number of collagen-containing vacuoles during the course of fibrosis.

Fat-storing cells, transitional cells, myofibroblasts and fibroblasts of the rat liver are fibroblast-like cells which may be closely related. The condition of the liver parenchyma may determine the phenotypic expression of the fibroblast-like cells. In normal liver, fat-storing cells are perisinusoidal cells that participate in the storage of vitamin A and in the maintenance of the sinusoidal structure of the liver lobules. After an insult to the liver, fat-storing cells of injured areas become activated and may be transformed into transitional cells that appear to have phenotypical and biochemical features of fibroblasts

and myofibroblastic cells (see chapter 1.4.4). In chapter 5A, we found that newly administered vitamin A can also be stored in fibroblasts of the fibrotic septa induced by CCl_4, which provides new evidence for the idea that these cells are derived from fat-storing cells. Vitamin A changed not only the phenotype expression of fibroblasts but also reduced collagen depositions of the fibrotic liver indicating a reduced capacity of these cells to synthesize or increased capacity to catabolize collagen.

The close relationship between fat-storing cells, myofibroblasts and fibroblasts is also confirmed by in vitro studies. Fat-storing cells, with des^+sma^- immunoreactivity, isolated from normal rat liver transform spontaneously into myofibroblastic cells in culture, expressing both desmin and SMA (29). Moreover cultured skin fibroblasts express SMA (30) indicating that the acquisition of myogenic characteristics is stimulus-dependent. Culture conditions stimulate cell division and transformation including synthesis of extracellular matrix proteins.

The results in chapter 6 are in line with the idea of phenotypic modulation of fibroblast-like cells. Transitional cells appeared both along the sinusoids and in the scars, indicating the transformation of fat-storing cells or myofibroblasts. Furthermore, proliferation of des^+sma^- or des^+sma^+ cells was observed in the degenerating area around the central veins and in septa between adjacent central veins of the liver resulting in an increase of des^-sma^- cells during the course of hepatic fibrosis indicating phenotype modulation of liver fat-storing cells, transitional cells and myofibroblasts into fibroblasts.

In conclusion, the results suggest that the des^-sma^- fibroblasts of well-developed fibrotic septa are derived from both des^+sma^+ myofibroblasts and des^+sma^- fat-storing cells. During the early formation of septa, both myofibroblasts and fat-storing cells divide and transform. Locally increased collagen type IV deposition may be one of the earliest phenomena in the formation of septa.

8.5 CONCLUSIONS

The experiments with rats have resulted in some new insights into the pathogenesis of alcoholic liver disease. Through long-term experiments with ethanol in rats, in which other factors like dietary components, energy intake, vitamin A suppletion and genetic constitution could be varied, it became clear that the effects of alcohol on the organism as a whole, and on the liver specifically, are dependent on these factors.

In the rat, chronic excessive alcohol consumption alone does not necessarily lead to liver damage, even in life long studies. The results from this thesis indicate that, apart from clinicol-chemical parameters, the overall condition and dietary status should give an insight in risk calculation for liver damage in individual alcoholics. Malnutrition and

underfeeding are important cofactors, but also overfeeding can contribute to negative long-term effects of alcohol on the body.

The presence of other hepatotoxic factors, like certain drugs or (excess) retinoids, probably increases the risk of liver disease from alcohol abuse. Short-term intervention studies with chemically induced liver fibrosis showed some useful ways to influence progression and repair of fibrosis by means of dietary variation. Especially retinoids (vitamin A) and β-carotene show beneficial effects, although toxic interactions of retinoids with ethanol should be taken into account.

With the results from these experiments, we are able to create criteria for the recognition of alcoholics with an increased risk to developing irreversable liver damage. Furthermore, the possibility of the prevention of liver damage by means of dietary adjustment can be assessed. The clear effects of nutrition on the genesis and repair of liver fibrosis indicate that specific attention should be given to nutritional status and intake (low fat, sufficient micronutrients, low calorie, ß carotene, lecitin). In this view, high doses of retinoids with toxic side effects, but also vitamin A depletion of the liver, should be considered as possible cofactors. After removal of the primary toxin (alcohol), the risks of toxic interactions are reduced and therapeutic support with vitamin A suppletion can be considered, but preferably in the form of β carotene.

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SAMENVATTING

Door een sterke toename van het alcoholgebruik per hoofd van de bevolking in de westerse maatschappij in de laatste decennia is het aantal mensen dat lijdt en overlijdt aan alcoholische leverziekte aanzienlijk toegenomen. In Nederland is de consumptie per hoofd van de bevolking gestegen van 2.1 liters zuivere alcohol in 1950 naar 8.0 liters in 1991. Met de toename van het alcoholgebruik in Nederland steeg ook het aantal patiënten met alcohol-gerelateerde ziekten, hetgeen resulteerde in een toename van de mortaliteit ten gevolge van deze ziekten tussen 1960 en 1985 met 150%. Met name de mortaliteit ten gevolge van alcoholische leverziekten is hoog. Behandeling van alcoholische leverziekten is in de praktijk moeilijk. Alcoholische leverpatienten genezen zelden, mede doordat onthouding niet vaak succesvol is. Het is van belang de mechanismen van de toxiciteit te leren kennen, maar vooral ook de (beheersbare) factoren die bepalend zijn voor het optreden van leverziekten ten gevolge van overmatig alcoholgebruik.

Het onderzoek beschreven in dit proefschrift is gericht op de ontstaansmechanismen van alcoholische leverziekte met als doel het herkennen van risicofactoren die bijdragen aan het ontstaan van ernstige irreversibele afwijkingen. Het onderzoek is uitgevoerd in de rat waarbij gebruik is gemaakt van verschillende korte en lange termijn modellen, waarbij naast alcohol ook andere hepatotoxische stoffen, zoals het xenobioticum carbontetrachloride (CCl_4), zijn bestudeerd. De rol van voedingsfactoren (o.a. vet en vitamine A), energie-inname, genetische constitutie en de participatie van verschillende type levercellen in het ontstaan van leverfibrose werden onderzocht. Aan de hand van het CCl_4-model in de rat werd de potentie van vitamine A en β-caroteen om leverfibrose te voorkomen c.q. genezing te verbeteren onderzocht.

Het onderzoek met ratten was in eerste instantie gericht op het bestuderen van de pathogenese van alcoholische leverfibrose, waarbij werd uitgegaan van het door de groep van Lieber beschreven model waarbij alcohol in een vloeibaar dieet werd toegediend. Dit semi-synthetische alcohol-dieet bleek in tegenstelling tot de resultaten van Lieber in geen van de drie gebruikte rattestammen leverfibrose te induceren, ondanks verlenging van de behandelingsperiode van 9 tot 16 maanden. Lichte leverschade met verhoogde transaminasegehaltes van het serum, alsmede stamafhankelijke veranderingen in het lipide- en lipoproteïnemetabolisme en extrahepatische afwijkingen (waaronder staar) werden wel in de met alcohol behandelde dieren gevonden. Het metabolisme en de stapeling van vitamine A in de lever werden, in tegenstelling tot door anderen gepubliceerde resultaten, nauwelijks door alcohol beïnvloed.

Het model van Lieber in de door ons bestudeerde rattestammen kon niet worden gereproduceerd, ondanks aanzienlijke verlenging van de behandelingsduur. Hoewel er

bij de met alcohol gevoerde dieren opvallend veel, ook ernstige, infecties in verscheidene andere organen dan de lever werden aangetroffen, welke mogelijk invloed hebben op het effect van alcohol op de lever, was het niet zinvol dit model verder voor pathogenetische studies te gebruiken.

Een deel van het onderzoek is vervolgens gericht op een aantal andere mogelijkheden om in de rat met behulp van alcohol leverfibrose te induceren. Hierbij zijn voedingsfactoren (ondervoeding, vitamine A status) en wijze van toediening gevarieerd.

Langdurige inname van een eiwitarm en vetrijk dieet, marginaal in micronutriënten, leidde op zich tot de inductie van steatose en leverfibrose. Deze afwijkingen traden versneld en sterker op als dit dieet wordt gecombineerd met langdurige, overmatige alcoholinname. Ook uit deze lange termijn experimenten bleek dat, ondanks duidelijke effecten van alcohol op de algehele conditie en op de histopathologie van andere organen, alcoholische leverschade slechts optrad onder condities die op zich ook al tot leverafwijkingen leidden. Wel bleek alcohol deze effecten aanzienlijk te versterken.

Alcohol-geïnduceerde veranderingen in levermorfologie en leverfysiologie in de rat bleken sterk afhankelijk van de samenstelling van het dieet. Bij een uitgebalanceerd standaarddieet leidde permanente toediening van drinkwater met 25% alcohol (v/v) bij de rat nauwelijks tot steatose, hepatitis of leverfibrose of andere leverafwijkingen. Wel gaf alcohol bij deze dieren een aanzienlijke reductie van de levensduur. Staar en enkele andere typische ouderdomsverschijnselen traden vervroegd en veelvuldiger op, zij het in afhankelijkheid van de genetische achtergrond. Indien gecombineerd met een gerestricteerde voerinname tot 60% van de ad libitum inname (calorische restrictie), bleken de gevolgen van alcohol veel minder, was de levensduur langer en de frequentie van verouderingsgerelateerde afwijkingen lager dan in ad libitum gevoerde alcoholratten. Deze resultaten duiden erop dat een belangrijk deel van de schadelijke effecten van alcohol verlopen via mechanismen die ook een rol spelen bij veroudering.

Door herhaalde toediening van CCl_4 kan in de rat in 4 weken leverfibrose worden geïnduceerd. Deze gaat gepaard met parenchymale celvervetting, celnecrose en hepatitis. CCl_4 induceert een vorm van leverfibrose die grote overeenkomsten vertoont met door alcohol geïnduceerde fibrose. Dit experimentele diermodel is gebruikt om de relatie tussen leverfibrose en vitamine A verder te onderzoeken. Hierbij is aangesloten bij de sterke aanwijzingen dat de fat-storing cellen, die vitamine A opslaan, een essentiële rol spelen bij de bij fibrose optredende vorming van overmatig bindweefsel. In dit model is nagegaan in hoeverre vitamine A en β-caroteen in staat zijn fibrotisering van de lever te voorkomen, c.q. het herstel van fibrose te bevorderen.

Bij ratten waarbij vooraf met CCl_4 een milde leverfibrose was opgewekt, bleek dat een behandeling met alcohol van enkele weken de ernst van de fibrose versterkte. Dit impliceert dat alcohol synerchistisch werkt met CCl_4. Veel van de toxische effecten van alcohol op de lever kunnen mogelijk worden teruggevoerd op een samenspel van alcohol

met andere stoffen, waarvan het hepatotoxische effect door alcohol wordt gepotentiëerd.

De vitamine A leverstatus bleek een belangrijke invloed te hebben op de ernst van de door CCl_4-geïnduceerde leverfibrose. Een lager vitamine A gehalte ging gepaard met een ernstiger mate van leverfibrose.

Er werden echter ook toxische effecten van vitamine A gevonden, indien het, voorafgaande aan of tijdens de CCl_4-behandeling, in hoge doseringen in de vorm van retinylesters werd toegediend. Een groot deel van de dieren overleed tijdens de gecombineerde behandeling, terwijl alle dieren die alleen CCl_4 of alleen vitamine A toegediend kregen de behandeling overleefden. Vitamine A had daarentegen een gunstig effect bij reeds ontwikkelde leverfibrose: Nabehandeling met een meermalige, hoge dosering van vitamine A gaf een aanzienlijke reductie van de leverfibrose die door CCl_4 was opgewekt. Vitamine A remt mogelijk de collageen synthese van fat-storing cellen of andere fibroblastachtige cellen en beïnvloedt zeer waarschijnlijk de differentiatie en proliferatie van fibroblastachtige cellen in de lever. De resultaten van aanvullende experimenten duiden erop dat ook β-caroteen (provitamine A) de ernst van de leverfibrose kan verminderen. In tegenstelling tot vitamine A is β-caroteen in overeenkomstige concentraties niet toxisch in combinatie met CCl_4.

Gezien de belangrijke rol die wordt toegeschreven aan "fat-storing" cellen en andere fibroblastachtige cellen bij de toegenomen collageen depositie in de fibrotische lever, is er onderzoek verricht naar het verschijnen en de verdeling van fibroblastachtige cellen ("fat-storing" cellen, "overgangscellen", myofibroblasten en fibroblasten). Dit onderzoek werd uitgevoerd met behulp van het CCl_4-model onder gebruikmaking van immunohistochemische technieken en ultrastucturele karakterisatie. Het bleek dat zowel "fat-storing" cellen als myofibroblasten een rol spelen in de initiële fase van leverfibrogenesis. Myofibroblasten alsook "fat-storing" cellen proliferen en gaan sterk lijken op fibroblasten. De resultaten suggereren dat de "overgangscellen" en de fibroblasten in de fibrotische septa van cirrhotische levers afkomstig zijn van zowel myofibroblasten alsook "fat-storing" cellen. De verschillende fibroblastachtige cellen in de lever zijn waarschijnlijk aan elkaar gerelateerd.

Dit proefdieronderzoek heeft een aantal interessante nieuwe bevindingen opgeleverd van zeer uiteenlopende aard. Via lange termijn experimenten, waarbij naast de alcoholtoediening ook andere factoren, zoals voedingssamenstelling, energie-inname, vitamine A suppletie en genetische constitutie werden gevarieerd, kon worden vastgesteld dat de effecten van alcohol op het organisme in zeer belangrijke mate afhangen van deze factoren. Daarnaast is gebleken dat de ernst van de afwijkingen in de lever mede bepaald wordt door secundaire reacties op afwijkingen elders in het lichaam. Net als in de mens blijkt dat chronisch overmatig alcohol gebruik in de rat niet noodzakelijk tot het ontstaan van leverafwijkingen leidt. De bevindingen in dit proefschrift duiden erop dat

naast klinisch-chemische parameters ook de analyse van de algemene conditie en de voedingsstatus aanknopingspunten moeten bieden voor de inschatting van risico's van toekomstig leverlijden bij individuele drinkers. Ondervoeding of een slecht samengestelde voeding zijn belangrijke cofactoren, maar ook overvoeding kan bijdragen tot de nadelige lange termijn effecten van alcohol op het lichaam.

De aanwezigheid van andere potentieel hepatotoxische factoren, zoals bepaalde geneesmiddelen en hypervitaminose A, verhogen waarschijnlijk het risico van leverafwijkingen door alcoholgebruik in belangrijke mate. Korter lopende interventiestudies bij chemisch geïnduceerde leverfibrose toonden aan dat er goede mogelijkheden bestaan om via de voeding de progressie van fibrose en het herstel ervan gunstig te beïnvloeden. Met name retinoids (vitamine A) en β-caroteen hebben in dat opzicht veelbelovende effecten, hoewel het risico van toxische interacties bij retinoiden niet denkbeeldig is.

Uit dit onderzoek zijn derhalve verbeterde criteria voor het herkennen van alcoholdrinkers met verhoogd risico voor het ontwikkelen van irreversibele leverschade te formuleren, terwijl ook de mogelijkheden om toekomstig leverfalen te voorkomen via voedingsinterventie beter kunnen worden ingeschat. De duidelijke, deels tegengestelde effecten van vitamine A op het ontstaan en het herstel van leverfibrose geven aan dat aandacht voor de vitamine A status en inname is geboden. Hierbij dienen zowel overdosering van vitamine A met toxische interacties, als een tekort aan vitamine A voorraad in de lever als mogelijke cofactoren te worden beschouwd. Na het wegnemen van het primaire toxine (abstinentie van alcohol) zijn de risico's van toxische interacties waarschijnlijk zo gering dat therapeutische ondersteuning met suppletie van vitamine A kan worden overwogen.

NAWOORD

Het verrichten van wetenschappelijk onderzoek en het tot stand laten komen van een proefschrift is geen sinecure en zeker geen eenmanszaak. Vele personen waren betrokken bij de opzet, de uitvoering en de verwerking van de gegevens. In deze ben ik mijn collegae van het voormalige TNO Instituut voor Experimentele Gerontologie dank verschuldigd.

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CURRICULUM VITAE

Willem Friedrich Seifert werd geboren op 31 maart 1960 te Schiedam. Na het behalen van het diploma VWO in 1979 aan het Goois Lyceum te Bussum, werd in hetzelfde jaar begonnen met de studie biologie aan de Rijksuniversiteit te Utrecht. In november 1982 deed hij kandidaatsexamen met als specialisatie medische biologie. In juni 1986 werd het doctoraalexamen afgelegd met als hoofdvakken Moleculaire Neurobiologie en Klinische Immunologie en als bijvak Virologie. Van mei 1986 tot november 1989 was hij als wetenschappelijk onderzoeker werkzaam bij de afdeling Orgaan- en Celfysiologie, sectie Voeding en Veroudering, van het TNO Instituut voor Experimentele Gerontologie te Rijswijk, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Van november 1989 tot juni 1992 was hij hoofd van de sectie Biotechniek van het TNO Instituut Toxicologie en Voeding te Zeist. Sinds juni 1992 is hij als wetenschappelijk onderzoeker verbonden aan de faculteit der Geneeskunde van de Katholieke Universiteit Nijmegen, afdeling Algemene Chirurgie van het St. Radboudziekenhuis.

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