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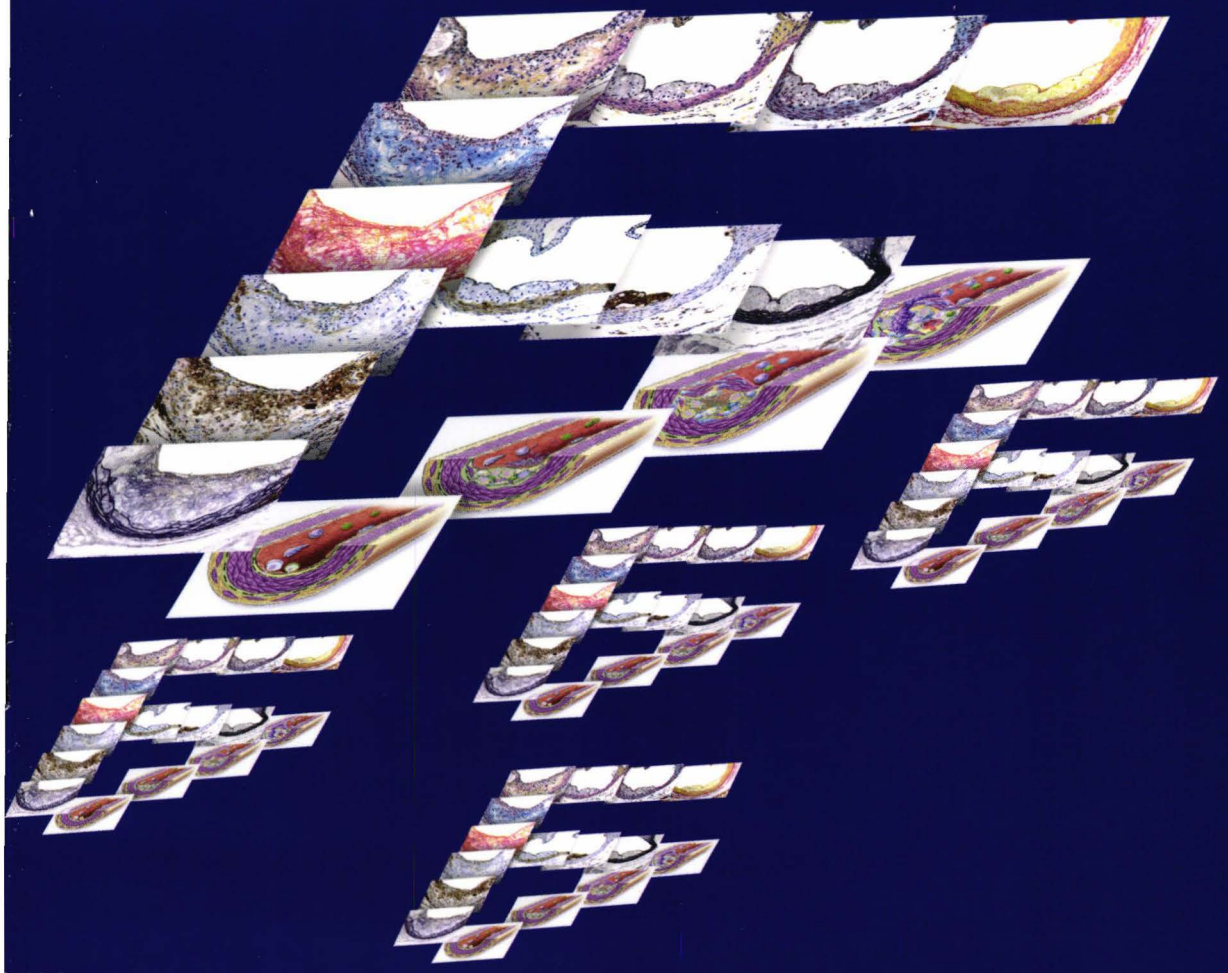

Farhad Rezaee

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Whoever is wise, is competent,
The heart of the old remains young through knowledge.

Ferdowsi (1088-1170)

All Adam's race are members of one frame
Since all, at first, from the same essence came.
When by hard fortune one limb is oppressed,
the other members lose their wanted rest.
If thou feel'st not for others' misery,
A son of Adam is no name for thee.

Saadi (1213-1291)

برای نسرین

For my Ariafar
Parents

CONTENTS

Chapter I

General introduction

INTRODUCTION

Cardiovascular disease and particularly atherosclerosis is the leading cause of mortality and morbidity in Western countries. Atherosclerosis is a disease of multifactorial etiology involving the interaction between genetic and environmental factors modulating the functions of various cell types and inflammatory molecules within the vessel wall. The lipoprotein-metabolism, coagulation cascade, fibrinolytic system, and vascular wall play a central role in atherosclerosis **(1-5).**

INTACT VASCULAR WALL

An intact arterial wall is composed of three layers: the intima (tunica intima), the media (tunica media) and the outer adventitia (tunica externa) **(6).** The intima is defined as the region of the arterial wall from and including the endothelial surface at the lumen to the luminal margin of the media. The internal elastic lamina (lamina elastica interna) is an elastic membrane, which separates the intima from the media. The latter is composed of smooth muscle cell (SMC) layers and is embedded in an interstitial matrix. This matrix mainly consists of elastin, collagen (particularly type I, III, and V), fibronectin, and thrombospondin. The elastic membrane consists of laminin, collagen type IV, heparan sulphate proteoglycans, and entactin (nidogen). The media is separated from the adventitia by the external elastic lamina (lamina elastica externa), composed of elastin and collagen. The adventitia consists of elastin fibers, collagen, and fibroblasts.

ATHEROSCLEROSIS

The word atherosclerosis is derived from the Greek words for fat-rich (atheros) and hardening (scleros). Atherosclerotic lesions have been divided into six types of increasing severity: type I initial, type II fatty streak, type III intermediate, type IV atheroma, type V fibrous plaque, and type VI complicated lesions **(7-9).** The terms early and advanced lesions are used for type I lesions and II and type IV-VI lesions respectively. Type III lesions represents the stage that forms a bridge between early lesions (particularly type II lesions) and the initiation of the advanced lesions (atheroma).

INITIAL, FATTY STREAK, AND PROATHEROMA

Type I lesions represent the initial changes and are characterised by an increase in the number of macrophages and the appearance of macrophages and T-lymphocytes filled with lipid droplets (foam cells) in the intima of the artery. Type II lesions include fatty streak lesions, the first visible lesions, and consist of layers of macrophage foam cells and lipid-laden smooth muscle cells and minimal coarse-grained particles and heterogenous droplets of extracellular lipid in the intima of an artery. Type III lesions are characterized by the lipid-laden cells of type II, dispersed collections of extracellular lipid droplets and particles that disrupt the coherence of some smooth muscle cells in the intima of the artery. The lipid pools are situated just below the layers of macrophages and macrophage foam cells, substitute intercellular matrix proteoglycans and fibers, and drive smooth muscle cells apart. Although initial and fatty streak lesions themselves are not associated with any risk of cardiovascular disease and can be generally found in children but may also occur in young adults, there is evidence that fatty streak lesions are prone to proceed to atheroma and more advanced lesions. The main growth mechanism of type I-IV lesions results from the lipid accumulation. Lesion types I through III do not thicken the arterial wall appreciably and therefore do not narrow the lumen or obstruct or modify blood flow. Thus, these three types of lesions are clinically silent.

ATHEROMA, FIBROUS PLAQUE, AND COMPLICATED LESIONS

Type IV lesions (atheromatous plaque) are composed of a dense accumulation of extracellular lipid of type III lesions, which occupies an extensive but well-defined region of the intima. This kind of accumulation of the extracellular lipid is referred to as lipid core. This lipid core results in severe intimal disorganization and thickens the artery wall. Between the lipid core and the endothelial surface, the intima consists of macrophages, smooth muscle cells, lymphocytes, mast cells, and proteoglycans. When a lipid core undergoes an increase in fibrous connective tissue (mainly collagen), the lesion is classified as a type V lesion (fibrous plaque). A fibrous plaque is characterized by a core containing necrotic debris, a-cellular lipid, and collagen encapsulated in the connective tissue at the base of the plaque, adjacent to the media. The plaque is covered with a fibrous connective tissue cap, which contains smooth muscle cells, macrophages and T-lymphocytes. Type V lesions also contain a calcium

precipitation and elastic fibers. Therefore, type V lesions are also referred to as calcified lesions, fibroatheroma lesions, and / or fibrotic lesions. An atheromatous and fibrous plaque may develop fissures or disruptions at the lesion surface, hematoma or hemorrhage, and/or a thrombus. For this reason they are clinically overt, though otherwise clinically silent. Atheromatous and fibrous plaques with one or more of these additional features are defined as type VI or complicated lesions. Atheromatous lesions are especially prone to disruptions of the lesion surface including fissures or tears, and ulcerations. Fissures probably reseal, incorporating hematomas and thrombi into the lesion. The components of lesions (i.a. inflammatory cells, released toxic substances, proteolytic enzymes) associated with thrombus formation cause or facilitate disruptions of the lesion surface including fissures. Some thrombi continue to enlarge and occlude the lumen of an artery and are often prone to rupture, and may lead to e.g. myocardial infarction. Type VI lesions are often very obstructive and symptom-producing, and therefore generally clinically overt. The main growth mechanism of type IV lesion results from lipid accumulation and smooth muscle cell increases. The main growth mechanism of Type V lesions is caused by accelerated smooth muscle cells and the increase of connective tissues (mainly collagens). Thrombosis and /or hematoma are the main cause of the growth mechanism of the complicated lesions.

DEVELOPMENT OF ATHEROSCLEROSIS

In the 19th century, Rokitansky and Virchow formulated respectively two hypotheses to explain the pathogenesis of atherosclerosis, The "incrustation" **(10)** and the "lipid" **(11)** hypotheses. The former modified by Duguid **(12),** suggested that intimal thickening is caused by fibrin deposition, with subsequent organization by fibroblasts and secondary lipid accumulation. The lipid hypothesis suggested that lipid in the arterial wall represented a transduction of blood lipid, which subsequently formed complexes with acid mucopolysaccharides; lipid accumulated in arterial walls because mechanisms of lipid deposition predominated over those of removal. The integration of these two hypotheses **(13)** subsequently led to a more complex "response to injury" hypothesis developed by Russell Ross **(2, 4)**. In this hypothesis three different phases are involved in the development of atherosclerotic lesions: fatty streaks, plaque formation, and complicated stages (figure 1 A-D).

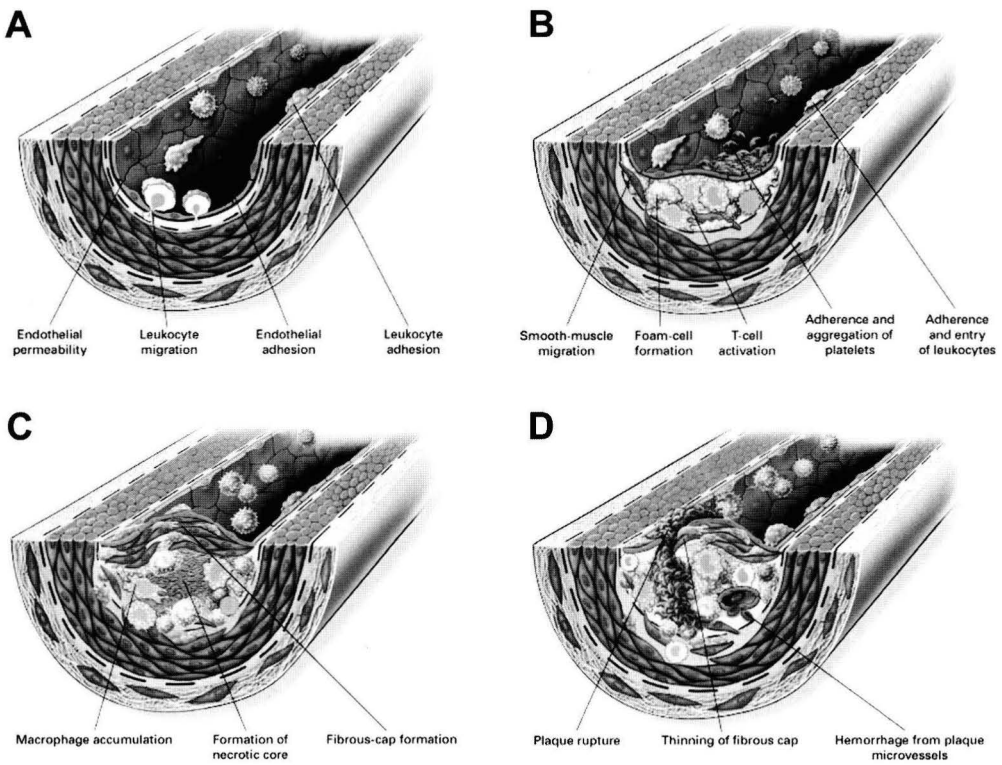

Figure 1. Development of human atherosclerosis.
Response-to-injury hypothesis. Endothelial dysfunction (**A**), Fatty-streak development (**B**), Complicated plaque formation (**C**), and unstable plaque formation (**D**) in atherosclerosis (adapted from reference **4**).

FATTY STREAK FORMATION

The development of atherosclerosis in humans is a slow process. The process is initiated early in life, but takes decades to develop mature plaques with clinical symptoms. On the basis of the "response to injury" hypothesis endothelial dysfunction is the first step in atherosclerosis (figure 1 A). The damage to the endothelium can be caused by various factors like mechanical stress, elevated low density lipoprotein cholesterol (LDL-C), elevated plasma homocysteine concentration, free radicals, cigarette-smoking, toxins, viruses, microorganisms, and the combination of these or other factors. When plasma LDL levels are elevated these lipoproteins can accumulate in the vascular wall where they become modified either enzymatically or non-enzymatically. In response to the modified lipids, endothelial

cells start to express adhesion receptors, which recruit circulating monocytes / macrophages and T-lymphocytes. Chemotaxis of these cells is mediated by several growth factors. These cells migrate through endothelial cells into the intima under the influence of various growth factors and chemoattractants produced by the endothelium, smooth muscle cells, monocytes/macrophages, and T-lymphocytes. In this phase the monocytes differentiate into macrophages. The macrophages start to scavenge the modified lipoproteins. The up-take of modified lipoproteins by macrophages can lead to a massive intracellular deposition of cholesterol. These lipid-laden cells are referred to as foam cells. Intimal smooth muscle cells together with the macrophage foam cells then form a fatty streak (figure 1 B).

PLAQUE FORMATION

Under the influence of diverse factors such as cytokines, growth factors and other molecules that are locally released by endothelial cells, macrophages, and smooth muscle cells attract more cells into the lesion. Here smooth muscle cells at the lesion site also proliferate, migrate, and secrete abundant collagen-rich extracellular matrix, giving rise to an elevated intimal lesion: the mature atherosclerotic plaque. This plaque is covered with a fibrous connective tissue cap, which contains smooth muscle cells, macrophages, and T-lymphocytes (figure 1 C).

COMPLICATED LESIONS

Mature atherosclerotic plaque consists of two main components: soft, lipid-rich atheromatous and hard, collagen-rich sclerotic tissue secreted by smooth muscle cells. The latter is the most voluminous, but it is relatively innocuous because of an abundance of collagen. Collagen makes the fibrous cap hard and thick, stabilizing the plaque against disruption and rupture. In contrast, soft lipid-rich atheromatous plaque (gruel) is separated from the vascular lumen by a cap of fibrous tissue, which is often thinnest at the shoulders. Thinning of the fibrous cap is apparently due to the continuing influx and activation of macrophages, which are capable of degrading extracellular matrix by phagocytosis or by secreting proteolytic enzymes, such as plasmin and metalloproteinases, which may weaken the fibrous cap. Thus, soft lipid-rich atheromatous plaque destabilizes the plaque, making it vulnerable and predisposes to rupture and thrombosis. Thus, disruption (rupture or fissure) of a

vulnerable or unstable plaque surface with a subsequent change in plaque geometry and thrombosis results in a complicated lesion (figure 1 D). This leads to further plaque growth and the increase of stenosis, resulting in acute occlusion with myocardial infarction.

ATHEROSCLEROSIS AND THROMBOSIS

Atherosclerosis without thrombosis is referred to as an uncomplicated plaque. Atherosclerosis in the form of lipid accumulation, macrophage infiltration and smooth muscle cell proliferation is in general a benign disease. It is thrombosis superimposed on mature plaques that converts a benign disease into life-threatening conditions. Most disturbed plaques are resealed by a small mural thrombus, and only sometimes a major luminal thrombus evolves. Fibrin(ogen) and their plasmin-generated degradation products with a variety of biological properties (chemotaxis, mitogen, increase in vascular permeability and angiogenesis), are found in the intimal and subintimal layers of the arterial vessel wall. Their local concentrations vary considerably, depending on the presence and severity of atherosclerotic lesions. This suggests a possible pathogenic role for intramural thrombi formed as a consequence of plaque fissure, which are readily incorporated into growing intimal lesions **(14-19).**
There are three major determinants of thrombosis (vessel, flow, blood), called Virchow's triad, that are of importance for thrombotic response to disruption / erosion of a plaque.
The amount and character of exposed thrombogenic material determines the severity of thrombotic response to disruption / erosion of a plaque **(20, 21).** Soft lipid-rich atheromatous plaque makes not only the plaques vulnerable to rupture that precipates luminal thrombosis, it also seems to be the most thrombogenic component (thrombogenic substrate).
Local flow disturbances can result from a severe stenosis at the rupture site and surface abnormality, which may activate platelets. Thus, the luminal thrombus formation increases with increasing severity of stenosis, possibily due to shear-induced platelet activation. Abnormalities of the exposed surface affect thrombogenicity **(20, 21).**
Fibrinolytic activity and thrombogenic factors such as plasma fibrinogen and platelet aggregation are associated with the development of acute myocardial infarction **(20, 21).**

Thus, thrombotic-thrombolysis balance at the time of plaque disruption also affects the outcome.

HAEMOSTASIS

The haemostatic mechanism can be delineated as a dynamic balance between two opposing processes, fibrin formation (coagulation) and fibrin dissolution (fibrinolysis) (figure 2). The former ensures that after tissue injury a fibrin clot is formed, which prevents bleeding, whereas, the latter cares for lysis of the fibrin clots after tissue repair **(22-27).** These two cascades are multicomponent enzyme systems. They also take part in a wide range of other functions such as brain function, reproduction, wound healing, infection, cardiovascular events, and cancer. In addition, both systems have also been involved in cellular migration and proliferation, and tissue remodeling, which are crucial mechanisms for the repair of blood vessels **(25-30).**

FIBRINOGEN ORGANIZATION

Fibrinogen is a plasma glycoprotein composed of 2 half-molecules, with each half-molecule containing three non-identical polypeptide chains: Aα, Bβ, γ with molecular weights 66.0, 52.0 and 46.5 kDa, respectively **(31-34).** These polypeptides are linked by 29 disulfide bonds **(35-37)** (figure 3). Each of the three polypeptide chains is encoded by a separate gene **(38-42),** located at the long arm of chromosome 4 ($4^{q23\text{-}q32}$) **(43).** The genes are arranged in the order of γ-Aα-Bβ, with the gene for the Bβ chain transcribed in the opposite direction **(44, 46).** Furthermore, it has been shown that there is an excess of Aα- and γ-chains in human hepatoma cells and that synthesis of the Bβ-chain is rate-limiting for the assembly and secretion of mature fibrinogen **(47-50).** Hepatic parenchymal cells synthesize fibrinogen and secrete it into the blood circulation **(51, 52).**

FIBRIN FORMATION

Prothrombin is converted to thrombin either by a series of reactions, involving factors VIII and IX (intrinsic pathway) or by tissue damage and factor VII (extrinsic pathway). The formation of insoluble fibrin (stable fibrin) is the ultimate step in the

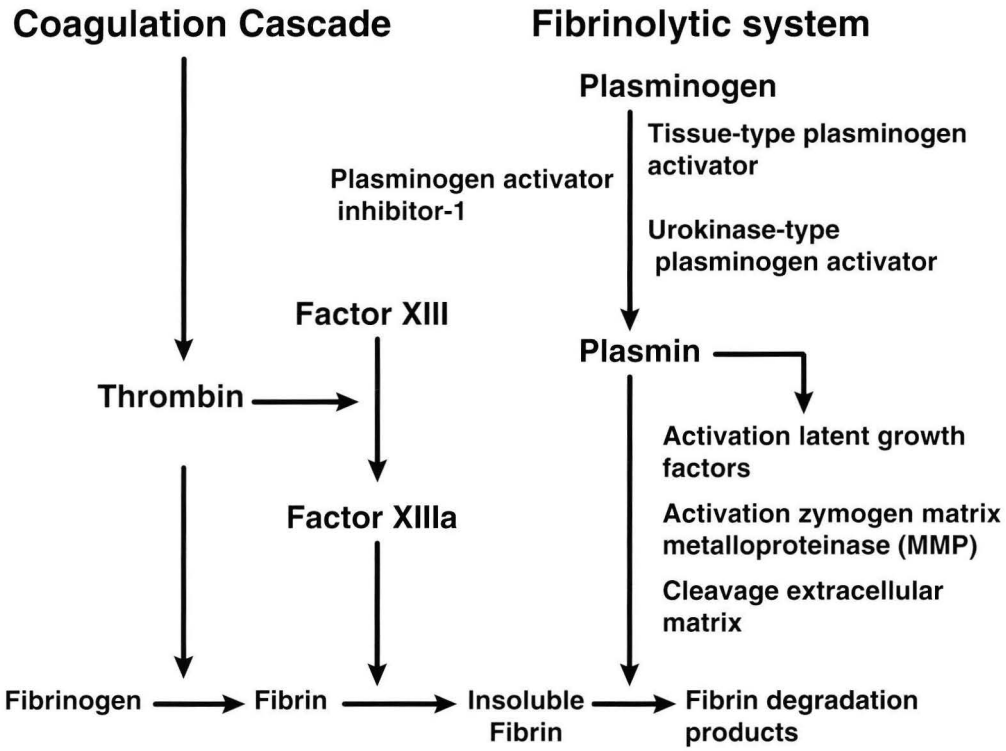

Figure 2. Schematic representation of the coagulation and fibrinolytic systems.

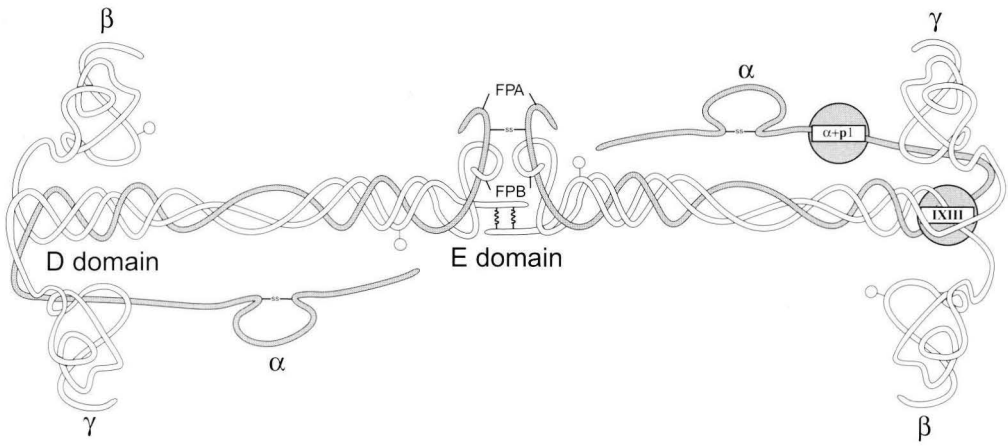

Figure 3. Schematic model of fibrinogen molecule.
The abbreviation used in this figure are: Fibrinopeptide A (FPA), Fibrinopeptide B (FBP), s-s (disulfide bonds), factor XIII (FXIII). (Adapted from reference **46**.)

coagulation cascade. Blood clots when fibrinogen, a highly soluble molecule, is converted to fibrin, by the action of thrombin. Thrombin catalyses the cleavage of the two fibrinopeptides A and B, with concurrent activation of coagulation factor XIII and generation of cross-linked fibrin polymers (insoluble fibrin) **(25-27, 53-57)** (figure 2).

FIBRINOGEN FUNCTIONS *IN VIVO*

To gain more knowledge about the possible roles of fibrinogen in the development of atherosclerotic plaques, it is necessary to understand its function ***in vivo*** **(56).**
The biological functions of fibrinogen can be divided into three major categories **(58-60). First**, fibrin seals the damaged blood vessels and restrains hemorrhage by participating in intravascular and extravascular coagulation. **Second**, fibrin forms a growth matrix for fibroblasts, platelets, macrophages, erythrocytes and other cells involved in wound healing and the recovery of tissue. **Finally**, fibrinogen is regarded as a major contributor to blood viscosity. Through these functions of fibrinogen, which play a role in hemostasis in healthy individuals, also may be involved in the development and progression of cardiovascular disease when fibrinogen levels are elevated. Thus, the homeostatic roles of normal fibrinogen levels can turn to pathologic phenomena and manifest as abnormal deposition of matrix, hyperviscous and hypercoagulable blood.

FIBRINOGEN, EPIDEMIOLOGY, AND ATHEROSCLEROSIS

The acute phase response is defined as the reaction of an organism to disturbances of its physiological homeostasis by tissue injury, infections and neoplastic growth **(61-63).** Fibrinogen is a positive acute phase protein, since its expression and secretion into the plasma is up-regulated during an acute phase response **(61-67).** Elevated plasma fibrinogen levels can occur as a result of a wide range of pathological circumstances. These conditions include cardiovascular disease and myocardial infarction, inflammatory reactions, infections, diabetes mellitus, traumatic cases and mental stress. Furthermore, increased plasma fibrinogen concentrations are associated with many lifestyle-related and metabolic factors such as cigarette-smoking, low-density lipoprotein cholesterol, and high-fat diets. **(68, 69).** Elevated plasma fibrinogen increases blood viscosity, which in turn, contributes to the pathology and severity of vascular disease. Hyperviscous blood increases shear stress on the

endothelium, leading to enhanced endothelial activation and the acceleration of atherosclerosis. Furthermore, extra force is needed to pump blood with high viscosity, which results in greater cardiac demand and worsens cardiac perfusion and angina symptoms in patients with ischemic heart disease. **(70-72).**

Elevated plasma fibrinogen has been shown in numerous epidemiological studies to be positively associated with a greater occurrence of cardiovascular disease **(73-81)** and atherosclerosis **(82-85)**. Some studies suggest that raised fibrinogen together with raised serum cholesterol increases the risk of cardiovascular disease to a greater extent than the other individual risk factors **(79)**. Furthermore, the risk associated with hypercholesterolemia remains low when combined with low fibrinogen concentrations **(79).** To date, a causal relationship between increased plasma fibrinogen and atherosclerosis is uncertain. This uncertainty is enhanced by the fact that fibrinogen is an acute phase protein with concentrations rising in response to inflammation **(61-67)**. Since atherosclerosis itself has often an inflammatory component **(1-4, 86)**, it is believed that atherosclerosis may elevate plasma fibrinogen concentrations. Data from the literature suggest that a critical role exists for inflammation in both atherogenesis and acute thrombosis **(4, 87, 88).** Whether fibrinogen may be considered as a simple marker or a real cause of atherosclerosis remains to be explored.

PLASMINOGEN / PLASMIN SYSTEM

The plasminogen / plasmin system that lyses fibrin is responsible for the counterbalance of the fibrin deposition. The fibrinolytic process is initiated by the conversion of plasminogen (a proenzyme) into plasmin (an active enzyme) via activators, such as tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The plasminogen activators are counteracted by plasminogen activator inhibitors (PAI) such as PAI-1 **(23, 25-29, 89, 90)** (figure 2), and plasmin by inhibitors such as α_2-antiplasmin and α_2-macroglobulin. Plasmin has a broad substrate specificity and degrades fibrin and other extracellular matrix proteins, and activates latent matrix metalloproteinases (MMPs) as well as growth factors **(25-28, 89-93).**

PLASMINOGEN / PLASMIN SYSTEM AND ATHEROSCLEROSIS

Epidemiological, genetic, clinical and molecular evidence suggests that the plasminogen / plasmin cascade participates in a wide range of pathological and physiological processes and it is suggested that plasminogen cascade is an important elements in atherogenesis and atherothrombosis formation. The plasminogen / plasmin activation cascade is involved in extracellular matrix proteolysis, cell migration and proliferation, tumor cell invasion and metastic spread, activation of latent growth factors as well as zymogens of the matrix-metalloproteinase enzymes, and generation of biologically active fibrin degradation products (FDPs) **(23, 25-30).** Thus, plasmin-mediated proteolysis could be involved in the progression of atherosclerotic lesions through several distinct mechanisms.

Increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) have been considered as a risk factor for myocardial infarction and recurrent infarction **(94-99),** and have been associated with the presence and evolution of coronary artery disease **(100-102).** However, the latter association has not been supported by other studies **(79, 103-105).**

A high level of tissue-type plasminogen activator (t-PA) antigen has been shown to be associated with the presence of coronary heart disease (CHD) **(79, 98, 106-109)** and to present a great risk of future myocardial infarction **(79, 106)** and stroke **(107, 110, 111).** An increased induction of t-PA and urokinase-type plasminogen activator (u-PA) in plaques suggests a possible role for increased plasmin proteolysis in atherosclerosis **(25-28, 112-114).** Plasmin proteolysis could be involved in the neovascularization of plaques, plaque rupture stimulation, aneurysm formation, ulceration, and atherosclerosis **(112-114).**

TRANSGENIC MOUSE MODELS FOR STUDYING ATHEROSCLEROSIS

Heterogeneity in both genetic and environmental factors as well as their interaction hampers the identification of the individual genetic and environmental factors involved in the development of atherosclerosis in humans. The use of a suitable animal model facilitates the study of the effect of fibrinogen, t-PA and u-PA and PAI-1 on the development of atherosclerosis under well-defined genetic and well-controled environmental conditions. Two recently developed technologies, gene targeting (homologous recombination in embryonic stem (ES) cells) and transgenesis

(gene transfer) **(115-123)** has allowed the manipulation of the genetic balance of candidate molecules in mice in a controlable manner. Thus, transgenic mice models have proven to be useful tools for studying many aspects of human cardiovascular disease in a homogenous genetic and environmental background. To circumvent the above-mentioned limitations, which are inherent in studying the contribution of fibrinogen, t-PA, u-PA, and PAI-1 in the development of atherosclerosis in humans, we turned to the transgenic mice models. We chose apoE3-Leiden transgenic mice **(124)** as a model for assessing atherosclerosis, because the lipoprotein profiles in these mice are very similar to those found in humans. Furthermore, the plaque formation could be induced, modulated and measured reproducibly in these mice by variation of the diet, resembling the human situation **(125).** This mouse model was used as genetic background for mice deficient in t-PA, u-PA, PAI-1 **(126, 127),** and mice with an increased level of fibrinogen in plasma.

SCOPE OF THIS THESIS

Understanding the genetic basis and the environmental factors as well as their interaction that affect atherosclerosis is very important for designing appropriate preventive measures and therapeutic intervention strategies. From a practical viewpoint, the mouse has been suggested to be an ideal animal model for such studies, because it is genetically defined, readily available, cheap to maintain, and reproduces easily **(128, 129)**. In this thesis we designed primarily an animal study with various mouse strains differing in atherosclerosis susceptibility **(130-133)** aimed at providing an answer or providing new insight into the following questions: -the relation between genetic background and basal plasma fibrinogen levels; possible modification of plasma fibrinogen levels via diet, taking into account both the total amount of dietary fat and its composition; the possible role of the acute phase response in plasma fibrinogen levels; the role of transcription in the regulation of fibrinogen; the relation between the kinetics of the fibrinogen response induction and the degree of susceptibility to atherosclerosis **(chapter 2)**.

Since the epidemiological studies suggest that a high level of plasma fibrinogen plays a role in the severity of atherosclerosis, it is important to gain more knowledge about the regulation of fibrinogen. In addition, it has been suggested that synthesis of the

Bβ-chain is rate-limiting for the assembly and secretion of mature fibrinogen. We have therefore undertaken an *in vivo* study of fibrinogen Bβ-gene biosynthesis in the mouse.

We generated transgenic mice with overexpression of the fibrinogen Bβ-gene and determined whether the additional copies lead to an increased plasma fibrinogen level **(chapter 3).**

Although increased plasma fibrinogen levels are linked with the severity of atherosclerosis as shown by many epidemiological studies, they do not establish which comes first. Because fibrinogen is an acute phase protein and atherosclerosis is a disease with many characteristics of an inflammatory process, a high level of plasma fibrinogen may be considered either as a simple marker or as a real cause of atherosclerosis. Because the epidemiological studies are not able to answer this question unambiguously, a mouse model of hyperfibrinogenemia was generated by conventional transgenesis to distinguish these two possibilities. **Chapter 4** describes generation and characterization of transgenic mice with overexpression of all three genes of fibrinogen.

Transgenic mice with hyperfibrinogenemia were crossbred with atherosclerosis-prone mice, apoE3-leiden mice, to study the contribution of fibrinogen in the development of atherosclerosis **(chapter 5).**

There is no established evidence for the involvement of the plasminogen / plasmin system in the development of atherosclerosis, although epidemiological, genetic and molecular evidence suggest that the plasminogen / plasmin system could be involved in vascular disease.

To explore the hypothesis that variations in endogenous fibrinolytic activity might significantly alter the process of atherosclerosis, we examined the effect of genetic modification of PAI-1, u-PA, and t-PA expression in a well-established model for atherosclerosis, apoE3-Leiden mice. These atherosclerosis-prone mice were crossbred with mice, which are inactivated in PAI-1 (PAI-1 -/-), u-PA (u-PA -/-), and t-PA (t-PA -/-) genes. Subsequently, the genetic compound offspring evaluated for atherosclerotic progression on a mild atherogenic diet for 12 weeks **(chapter 6).**

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Chapter II

Effect of genetic background and diet on plasma fibrinogen in mice. Possible relation with susceptibility to atherosclerosis.

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ABSTRACT

Although many epidemiological studies suggest that elevated plasma fibrinogen concentrations form one of the most important independent risk factors in blood for cardiovascular disease and particularly atherosclerosis in humans, they do not prove a causal relationship.

To clarify the effect of genetic factors, diets and their interactions on plasma fibrinogen concentrations, we examined plasma fibrinogen levels in four strains of mice, which differ in their susceptibility to cholesterol-induced atherosclerosis. The mice were fed normal breeding chow or four different semi- synthetic diets for different time periods.

When maintained on basal diet, two strains 129/J and C3H/HeJ exhibited a significantly higher plasma fibrinogen concentration (2.1 and 1.9 mg/ml) than C57BL/6J and BALB/C strains (1.5 and 1.4 mg/ml). The strongest and most rapid (1 week) increase of plasma fibrinogen (by all semi-synthetic diets) and of the acute phase marker haptoglobin (only by atherogenic diet) is observed in C57BL/6J mice, which are known to be highly susceptible to diet-induced atherosclerosis. After a period of 8 weeks an increase in plasma fibrinogen of approximately 30-50 % was observed in all strains on semi-synthetic diets. No increase was observed in the fibrinogen Aα- Bβ- and γ-chain mRNA levels in the liver on the same diets. The fibrinogen mRNA levels were even decreased by approximately 20-50 % in all strains on an extremely atherogenic diet containing 1 % cholesterol, 15 % saturated fat, 0.5 % cholate (N).

Genetic background determines the plasma fibrinogen levels on basal diet; plasma fibrinogen levels are altered by diet, and the extent of the changes depends on the genetic background; surprisingly the increase of fibrinogen in plasma due to the diets is independent of transcription; the diet-induced increase of fibrinogen was very fast in the very high atherosclerosis-susceptible strain C57BL/6J having a low basal level and very slow in the very high atherosclerosis-resistant strain C3H/HeJ having a high basal level. It might be concluded that it is the kinetics of the response of fibrinogen to diet rather than the actual level, which relates to atherosclerosis susceptibility.

Keywords: Fibrinogen; Atherosclerosis; Transcription, Diet; Mouse strains.

INTRODUCTION

Elevated plasma fibrinogen has been shown in numerous epidemiological studies to be positively associated with a greater occurrence of cardiovascular disease **(1-6)** and atherosclerosis **(7, 8)**. The cumulative evidence suggests that raised fibrinogen together with raised serum cholesterol increase the risk of cardiovascular disease incidence as compared to the individual risk factors **(5)**. Furthermore, the risk associated with hypercholesterolemia remains low when combined with low fibrinogen concentrations **(5).** To date, a causative relationship between the increased plasma fibrinogen and atherosclerosis is uncertain. This uncertainty is enhanced by the fact that fibrinogen is an acute phase protein with concentrations rising in response to inflammation **(9, 10)**. Since atherosclerosis itself has often an inflammatory component **(11, 12)**, it is believed that atherosclerosis may elevate plasma fibrinogen concentrations. Data from literature suggest that a critical role exists for inflammation in both atherogenesis and acute thrombosis **(11, 13, 14).** Fibrinogen is a plasma glycoprotein, composed of three different polypeptides: Aα, Bβ, γ **(15, 16)**, encoded by three separate genes **(17, 18).** The genes are arranged in the order of γ-Aα-Bβ, with the gene for the Bβ-chain transcribed in the opposite direction **(19, 20)**.

When fibrinogen is synthesized in the liver both Aα-chains are intact, high molecular weight (HMW). In the circulation fibrinogen is partly changed and two low molecular weight (LMW and LMW') forms of fibrinogen are formed with a decreased clotting rate **(21-24)**. Thus, a changed ratio of the fibrinogen forms could play an important role in cardiovascular disease and particularly in atherosclerosis.

Human studies regarding the dietary effects on plasma fibrinogen levels gave conflicting results (for reviews see Vorster *et al* **(25)**, and Miller **(26)** and the references therein). However, there are no human studies available regarding the dietary effects on the kinetics of the response of fibrinogen or on the relation between the kinetics of fibrinogen response induction and the degree of susceptibility to atherosclerosis.

In this chapter we report a study with various mouse strains differing in atherosclerosis susceptibility **(27-30)** aimed at providing an answer or providing new insight into the following questions: the relation between genetic background and basal plasma fibrinogen levels; possible modification of plasma fibrinogen levels via diets, taking into account both the total amount of dietary fat and its composition; the

possible role of the acute phase response in plasma fibrinogen levels; the role of transcription in the regulation of fibrinogen; the relation between the kinetics of the fibrinogen response induction and the degree of susceptibility to atherosclerosis.

MATERIALS AND METHODS

Animals

Female mice of strains C57BL/6J, BALB/C, 129/J, and C3H/HeJ 8-10 weeks of age were purchased from Jackson laboratory (Bar Harbor, U.S.A.) and maintained in a temperature-controlled room, illuminated from 7 AM through 7 PM. All animals were allowed to adapt to the environment for at least 3 weeks prior to the study. Mice were given free access to food and water throughout the experiment. Weight gain and food intakes were monitored two times during the period of this study. The protocol of this study was approved by the University of Leiden Ethics Commission for research animals (UDEC).

Diet and feeding

Five diets (Hope Farms, Woerden, The Netherlands) differing in their atherogenicity were used in this study. The first was pelleted rodent chow (C) (standard mouse/rat diet), which served as a reference diet for each mouse strain. The four semi-synthetic diets were: a diet high in saturated fat (T), a diet high in unsaturated fat (X), a sucrose-rich diet (Z), and an extremely atherogenic diet containing high fat, high cholesterol, and 0.5 % cholate (N). The exact composition of these diets is given in table 1. All diets were fed to five groups of mice of each strain for different time intervals. Five groups of mice of each strain were randomly assigned to each diet.

Collection of blood and tissue

After a three-week adaptation period to environment on chow diet for baseline measurements, or after 1, 2, and 4 weeks on semi-synthetic diets whole blood was collected into chilled tubes coated with EDTA (Sarstedt, Etten-Leur, The Netherlands) from the mouse tail vein. After eight weeks, whole blood was collected into chilled tubes containing trisodiumcitrate (0.011 M final concentration) after anesthesia.

Plasma was prepared by centrifugation of whole blood at 3000 x g for 20 minutes at 4 °C and stored at -20 °C until analysis. Upon termination of the experiments, mice were sacrificed by cervical dislocation after which the livers were removed and placed directly into liquid nitrogen and stored at -70 °C until analysis.

	Type of diet			
Diet components	N	T	X	Z
Cocoa butter	15	15	-	-
Cholic acid	0.5	-	-	-
Cholesterol	1	-	-	-
Corn oil	1	1	16	5
Cornstarch	10	10	10	10.98
Sucrose	40.5	40.5	40.5	55.11
Cellulose	4.7	6.2	6.2	4.47
Casein	20	20	20	17.9

Table 1. Composition of the four semi-synthetic diets.
The basic diet, containing only sucrose and basic nutrients, is designated as the low fat and low cholesterol (Z) diet. The atherogenic diet (N) consists of the basic diet supplemented with cocoa butter (15 %, w/w), cholesterol (1 %, w/w) and cholic acid (0.5 %, w/w). The saturated fat diet (T) consists of the basic diet supplemented only with cocoa butter. The unsaturated fat diet (X) consists of the Z diet supplemented with corn oil (16 %, w/w). In addition, all diets contained 2 % (w/w) choline chloride, 0.2 % (w/w) methionine, vitamin and mineral mixture 5.1 % (w/w).

Plasma fibrinogen and haptoglobin analysis

Plasma (pooled plasma of each strain on each semi-synthetic diet) concentrations of haptoglobin were estimated by radial immunodiffusion, using a Nor Partigen kit (Dade Behring, Leusden, The Netherlands), according to the manufacturer's recommendations. Haptoglobin is a positive acute phase reactant and served as an inflammation marker in this study.

Mouse fibrinogen concentrations were measured by sandwich ELISA, using purified polyclonal rabbit anti-rat fibrin antibodies **(31)** for coating and purified polyclonal goat anti-mouse fibrinogen antibodies conjugated to peroxidase as second antibodies (Nordic, Tilburg, The Netherlands). The detection was performed by the immunoperoxidase procedure using tetramethylbenzidine (TMB) as a substrate (Organon Teknika, Boxtel, The Netherlands). The fibrinogen concentration of Pooled citrate plasma from mice was determined by gravimetrical analysis **(32).** This pooled

plasma was subsequently used as the standard in ELISA assays. The fibrinogen levels obtained with this ELISA compare very well with functional fibrinogen as measured with the Clauss method **(31).**

Northern blot analysis

Total RNA was isolated from livers of different mouse strains using the RNAzol™ B procedure (Cinna/Biotecx, Veenendaal, The Netherlands). RNA samples (10 µg per lane) were fractionated by electrophoresis on a denaturing agarose gel (Boehringen Mannheim, Almere, The Netherlands) (1.2 w/v %) containing 0.75 % (w/v) formaldehyde and subsequently transferred capillarly to Nylon Hybond N^+ (Amersham-Pharmacia, Roosendaal, The Netherlands) according to the manufacturer's instructions, and UV cross-linked. RNA blots were subsequently hybridized with [^{32}P]-labeled (Amersham-Pharmacia, Roosendaal, The Netherlands) probes of mouse fibrinogen Aα- Bβ- and γ-chain cDNAs, and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA **(33)** overnight at 65 °C in a solution containing 1M Na-Phosphate (pH 7.2), SDS (7 % w/v) and EDTA (1.25 mM), according to instructions of the Megaprime kit (Amersham-Pharmacia, Roosendaal, The Netherlands). The blots were exposed to a Fuji imaging plate type BAS-MP for 1 to 24 h. The intensity of the hybridization signal was quantified with a Phosphor-Imager (Fuji Fujix BAS 1000) and analyzed with the computer programs BAS-reader and TINA version 2.8 and 2.08c. The amounts of mRNA of fibrinogen were normalized to the levels of GAPDH mRNA.

SDS polyacrylamide gel electrophoresis and Western blot analysis

To determine the ratio of High Molecular Weight to Low Molecular Weight (HMW/LMW) fibrinogen in plasma, non-reduced 10 µl diluted plasma containing 40 ng fibrinogen was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli **(34)** using 5 % Tris-HCl ready gels (Bio-Rad, Veenendaal, The Netherlands). Proteins were subsequently transferred electrophoretically onto 0.45 µm Protan nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) in a semi-dry-blot apparatus (Pharmacia-LKB, Roosendaal, The Netherlands). The filters were subsequently blocked with

Tris-HCl (10 mM, pH 8.0), NaCl (150 mM), non-fat dried-milk powder (5 % w/v), (Carnation Nestle, Glendale, U.S.A.), and Tween-20 (0.1 % v/v) at 4 °C overnight, followed by incubation with polyclonal rabbit anti-rat-fibrin-monomer, goat anti-mouse IgG conjugated to peroxidase as secondary antibody, and 4-chloro-1-naphtol as detection **(35)**. The blots were subsequently scanned.

Reverse transcription PCR (RT-PCR)

The primers used for PCR reactions were derived from rat fibrinogen, rat haptoglobin, and mouse major urinary protein (MMUP) cDNA sequences (table 2), (Isogen, Utrecht, The Netherlands). The reverse transcription reaction was performed using 2 µg of total RNA isolated from mouse liver, 0.5 µg/µl oligo dT-primer, in the presence of 1 x first-strand buffer (0.25 M Tris-HCl, pH 8.3, 0.375 M KCl, 0.015 M $MgCl_2$), 0.01 M DTT, 200 units of moloney-murine leukemia virus reverse transcriptase (Gibco-BRL, Breda, The Netherlands), 40 units of RNasin (Promega, Leiden, The Netherlands) and 0.1 mM dNTP mix (Amersham-Pharmacia, Roosendaal, The Netherlands). The reaction was carried out at 42 °C for 1 h. 2 µl of the RT reaction was then denatured for 5 min at 95 °C in a 50 µl reaction volume containing 0.25 mM of each dNTP, 1 µM primers, 5 µl Taq PCR buffer and 5 units Taq DNA polymerase (Amersham-Pharmacia, Roosendaal, The Netherlands). Then PCR amplification was performed using 35 cycles under the following conditions: 1 min 95 °C, 1 min 60 °C, and 2 min 72 °C in a thermal DNA cycler machine (Perkin Elmer Cetus, Norwalk, U.S.A.). 50 µl reaction product was then analyzed on a 1.0 % agarose gel. The primer sets of rat fibrinogen Aα- Bβ- and γ-cDNA sequences generate fragments of approximately 1.55-kb, 1.4-kb, and 0.98-kb respectively. The primer sets of rat Haptoglobin and MMUP cDNA generate 410 and 520 bp fragments

cDNA	Forward primer 5'-3'	Reverse primer 5'-3'
RAT Fbg Aα	CGTGGCCCAAGAATTGTGGAGAC	AAGAGCGGATCTTGATATCAATGTC
RAT Fbg Bβ	GCCCGTGGTCATCGACCTGTTGAC	CCAGACACCACCGGGATGTTGC
RAT Fbg γ	CTTACCAAACCGACGTGGACAC	GTCATTGTCCCAGGTACTGAAGTGC
RAT HAP	CTTGGCAGGCCAAGATGATC	CTCACACTTCTCCTGGTCAG
MMUP	CTGCTGCTGCTGTGTTTGGG	GGAGGCAGCGATTGGCATTG

Table 2. Primer pairs used for PCR reaction.
Table abbreviations are fibrinogen (Fbg), haptoglobin (HAP), mouse major urinary protein (MMUP), complementary DNA (cDNA).

respectively. The PCR fragments were purified from the agarose gel using a gel extraction kit (Genomed-ITK, Uithoorn, The Netherlands). The purified fragments were subsequently cloned into pCR II vector using a TA cloning kit (Invitrogen, De Schelp, The Netherlands). The inserts were then excised from pCR II vector by digestion with *Eco*RI (Gibco-BRL, Breda, The Netherlands) and used as cDNA probes.

Statistical analysis

Results are reported as mean ± SEM. Statistical differences were determined by analysis of variance (ANOVA), using SPSS version 8.0 for Windows 95. Comparison of data from more than two groups was done by one-way ANOVA. Post hoc analysis of significance was made by Scheffe test. Independent sample t test was also used in one case. $P< 0.05$ was accepted as statistically significant.

Results

Plasma fibrinogen analysis

Different plasma fibrinogen levels were observed in four strains of mice, when fed basal diet (figure 1). Strains 129/J and C3H/HeJ exhibited significantly higher plasma fibrinogen levels (2.1 ± 0.07 and 1.9 ± 0.05 mg/ml) than strains C57BL/6J and BALB/C (1.5 ± 0.05 and 1.4 ± 0.04 mg/ml) respectively. To explore plasma fibrinogen level variations after the diets, plasma fibrinogen levels were measured after feeding the mice the basal diet and four semi-synthetic diets for different time periods (figure 2 A-D). After feeding mice the basal diet, we observed no changes in plasma fibrinogen levels at different time intervals. After a period of 8 weeks an increase of approximately 30-50 % of plasma fibrinogen was observed in all strains in response to all semi-synthetic diets as compared to basal diet. The highest plasma fibrinogen concentrations occurred in 129/J and C3H/Hej (2.9 mg/ml) strains when fed the T diet for a period of eight weeks.

Regardless of semi-synthetic diet type, a sharp rise in plasma fibrinogen of approximately 40 % was observed within one week in C57BL/6J mice (figure 2 A-D). A similar rapid response was seen for BALB/C mice only after consuming the N diet (figure 2 A). No changes were observed in plasma fibrinogen levels for C3H/HeJ

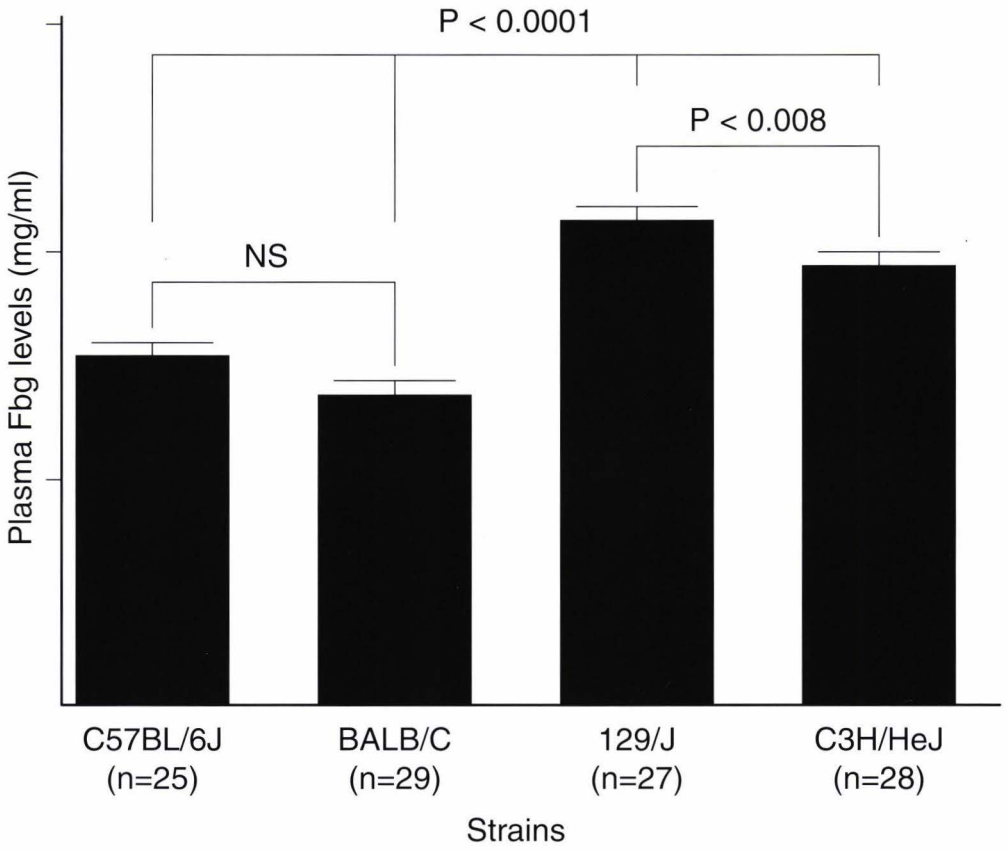

Figure 1. Plasma fibrinogen levels in different strains of mice fed chow diet for a period of three weeks.
The fibrinogen (Fbg) concentrations were determined in plasma of C57BL/6J, BALB/C, 129/J, and C3H/HeJ strains of mice fed basal chow (**C**) (standard mouse / rat diet) by an immunological method (ELISA). Mouse fibrinogen levels in plasma are expressed in mg/ml (mean ± SEM; n = number of mice). $P < 0.05$ was accepted as statistically significant (one-way ANOVA).

mice after being maintained for one week on any of the semi-synthetic diets. Strain 129/J mice showed a rapid decrease of plasma fibrinogen levels of approximately 25-35 % after 1 week when put on T, X and Z diets as compared to basal diet (figure 2 B - D).

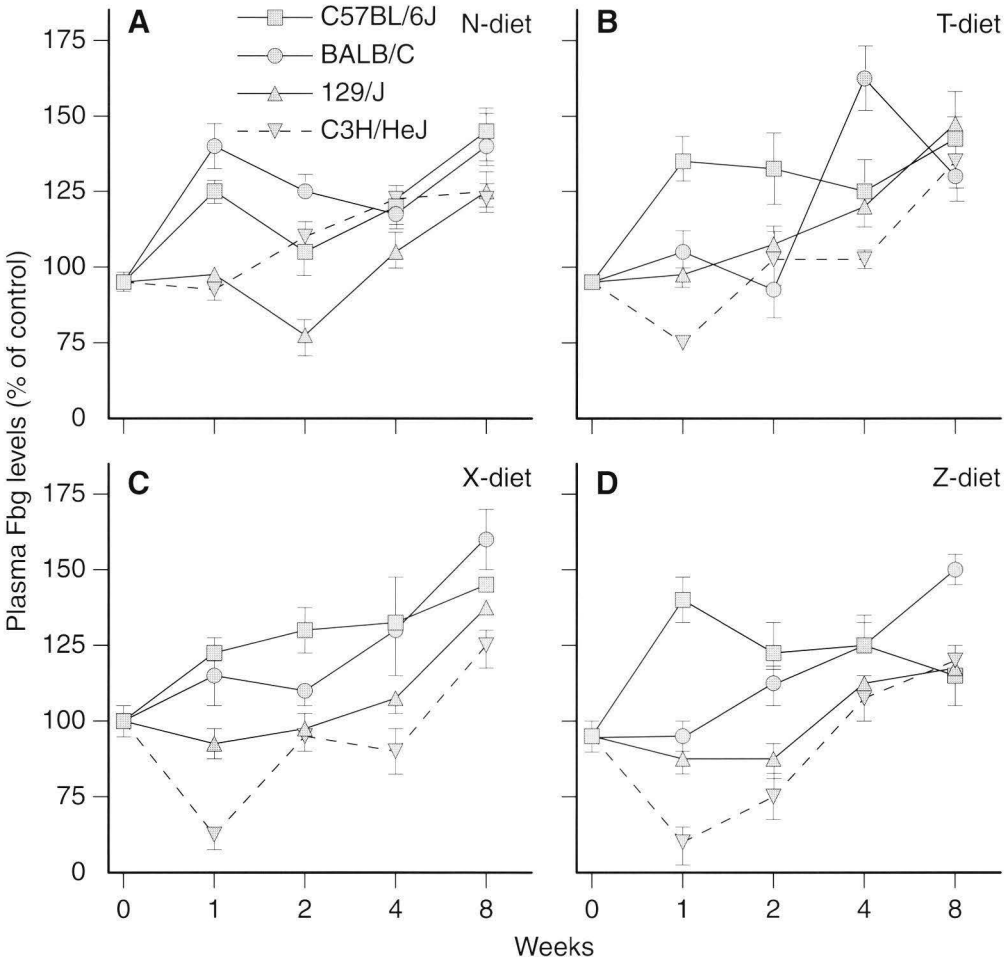

Figure 2. Plasma Fibrinogen levels in four different strains of mice fed five diets for different time intervals. Plasma fibrinogen (Fbg) was determined in C57BL/6J, BALB/C, 129/J, and C3H/HeJ strains of mice fed either basal chow (C) (standard mouse/rat diet) or saturated fat (T), unsaturated fat (X), sucrose (Z) and an extremely atherogenic (N) diet for different time intervals ranging from 1 to 8 weeks. No changes were observed for mouse strains with respect to plasma fibrinogen levels on chow diet during this period. The plasma fibrinogen baseline concentration (on chow diet) of C57BL/6J, BALB/C, 129/J, and C3H/HeJ strains of mice are 1.5, 1.4, 2.1 and 1.9 mg/ml respectively. Plasma fibrinogen levels are expressed as a percentage of levels in control mice on chow diet. Values for all measurements are expressed as the mean ± SEM of 5 to 8 mice per strain and diet type. $P < 0.05$ was accepted as statistically significant (one-way ANOVA).

Hepatic fibrinogen mRNA analysis

To determine whether the observed increase in plasma fibrinogen concentrations after feeding mice semi-synthetic diets for eight weeks was due to an increase in the hepatic fibrinogen mRNA levels, fibrinogen Aα-, Bβ-, and γ-chain mRNAs in the liver were measured (figure 3 A-D). Although plasma fibrinogen levels increased by

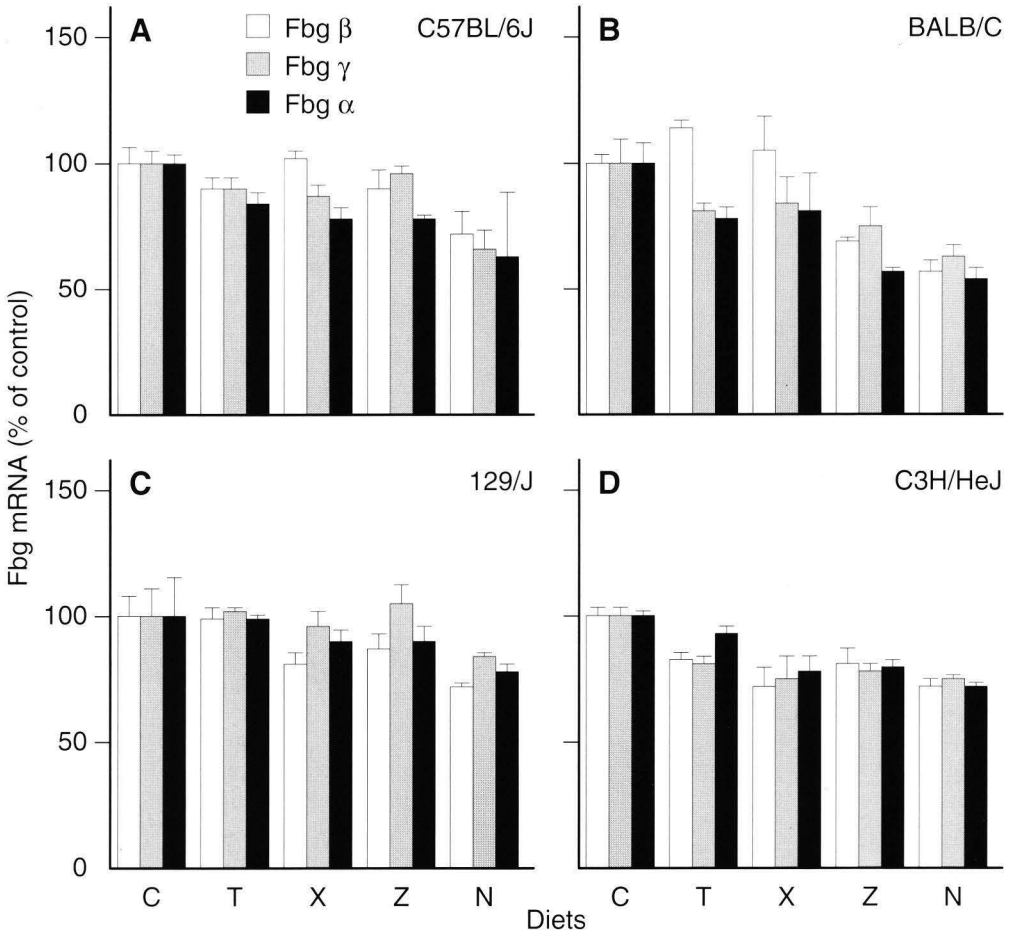

Figure 3. Hepatic fibrinogen mRNA levels in four different strains of mice fed five diets for a period of eight weeks. Fibrinogen Aα, Bβ, and γ, mRNAs (FbgAα, FbgBβ, and Fbgγ respectively) were determined by Northern blot in liver of C57BL/6J, BALB/C, 129/J, and C3H/HeJ strains of mice fed either basal chow (C) or saturated fat (T), unsaturated fat (X), sucrose (Z), and an extremely atherogenic (N) diet for a period of eight weeks. Mouse fibrinogen mRNA levels in liver are relative to an internal standard GAPDH and are expressed as a percentage of control mice on chow diet. Values for all measurements are expressed as the mean ± SEM of 5 to 8 mice per strain and diet type. $P < 0.05$ was accepted as statistically significant (one-way ANOVA). For details see Materials and Methods.

approximately 30-50 % in all strains in response to semi- synthetic diets over a period of eight weeks, no increase was observed in fibrinogen mRNA levels between strains as the result of semi-synthetic diets as compared to chow diet. After eight weeks' consumption of the N diet, all four strains of mice even exhibited a decrease of approximately 20-50 % in fibrinogen Aα-, Bβ-, and γ-chain mRNA levels as compared to basal diet.

Haptoglobin and mouse urinary protein analysis

To determine whether the acute phase response is responsible for the observed increase of plasma fibrinogen levels after feeding various diets, we measured two established acute phase markers haptoglobin (positive) and mouse urinary protein (negative), in mice either on semi-synthetic or basal diet. We observed that plasma haptoglobin levels did not change in any of the strains after being maintained on the T, X, and Z diets for various time intervals. These results indicate that there is no acute phase response in these mice as a result of the above-mentioned diets. In

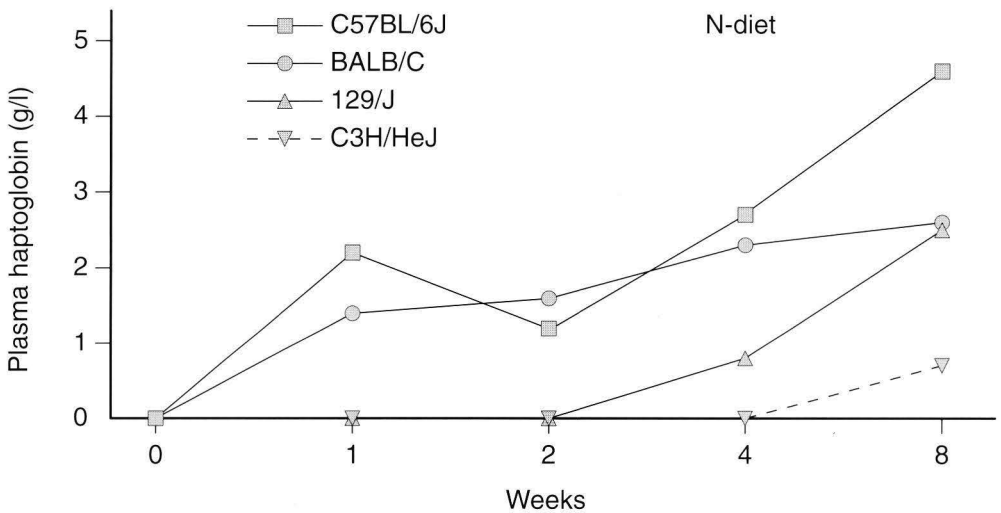

Figure 4. Plasma haptoglobin levels in four different strains of mice fed an extremely atherogenic diet for different time intervals.
The haptoglobin concentrations were determined in plasma of C57BL/6J, BALB/C, 129/J and C3H/HeJ strains of mice fed an extremely atherogenic (N) diet for different time intervals ranging from 1 to 8 weeks by radial immunodiffusion. Mouse haptoglobin levels in plasma are expressed in g / l. The number of mice ranged from 5 to 8 (mean ± SEM) per strain and diet and per time point. For details see Materials and Methods.

contrast, feeding an N diet resulted in the induction of an acute phase response in strains C57BL/6J, BALB/C, and 129/J, but hardly perceptible in C3H/HeJ. However, striking differences were observed between the strains with respect to the magnitudes and kinetics of acute phase response induction. As depicted in figure 4, a rapid increase of haptoglobin levels in plasma was observed for C57BL/6J (2.13 g/l), and at a lower degree for BALB/C (1.4 g/l) mice after one week of the N diet consumption. The acute phase response induction in 129/J and C3H/HeJ mice could be observed no earlier than at 2-8 weeks, and was considerably lower than in the other two strains. These results were confirmed by the quantification of either hepatic haptoglobin or mouse urinary protein mRNA levels after eight weeks on the same diets (data not shown).

HMW/LMW fibrinogen analysis

To determine whether the observed changes in fibrinogen concentrations are accompanied by a change in composition of the plasma fibrinogen, plasma HMW/LMW fibrinogen ratio was measured in a very rapidly responding (C57BL/6J) and a very slowly responding (C3H/HeJ) strain fed either basal or N diet. However, despite significant differences in absolute concentrations of plasma fibrinogen between C3H/HeJ and C57BL/6J strains, the ratio of HMW/LMW fibrinogen did not differ significantly (data not shown).

Discussion

An elevated plasma fibrinogen level has been identified in various epidemiological studies as an independent risk factor in cardiovascular events. We determined the effect of genetic factors, diet and the interactions of these on the fibrinogen synthesis and plasma levels in mouse strains, which differ in their susceptibility to diet-induced atherosclerosis.

Our results provide evidence for a significant genetic influence on fibrinogen concentrations in plasma. Two out of four strains of mice exhibited a significantly higher plasma fibrinogen level when maintained on a basal diet. The mechanism by which the genetic background affects the basal plasma fibrinogen levels in mice could be related to structural variations in the fibrinogen gene itself due to genetic background, or to other factors involved in transcription, translation, secretion, and /

or clearance of the plasma fibrinogen. Various studies regarding the relationship between the fibrinogen gene polymorphisms and the plasma fibrinogen levels have been reported in humans **(36-38).** Genetic variants of the Bβ-fibrinogen gene are associated with an increased plasma level of fibrinogen **(36)**. Most attention has been focused on the Bβ-fibrinogen gene ($G_{455} \rightarrow A$) polymorphism **(36-38),** which is strongly associated with higher plasma fibrinogen levels.

In addition to the genetic background, the effect of diet on plasma fibrinogen levels was investigated. Our results show that diet altered the plasma fibrinogen concentrations in mice and that the genetic background contributes to these diet-induced changes. Human studies regarding the dietary effects on plasma fibrinogen levels gave conflicting results (for reviews see Vorster *et al* **(25)**, and Miller **(26)** and the references therein), possibly due to interindividual variations. Our mouse study, however, was performed with genetically homogenous mouse strains, strict diet and environment control.

Furthermore in this study, we used different diets, which differ in their atherogenicity. The mechanism by which these diets possibly contribute to the development of atherosclerosis in C57BL/6J and not in C3H/HeJ mice could be due to the different production of lipoproteins in these strains. In response to the consumption of an atherogenic (N) diet, production of apoB-containing β-VLDL particles (cholesterol-containing VLDL) increases in the atherosclerosis-susceptible strain C57BL/6J mice **(39, 40).** This process can be further enhanced by the presence of cholic acid in the atherogenic diet (N diet). Cholic acid in diet facilitates the absorption of cholesterol in the intestine and leads to the suppression of bile acid synthesis, thereby shutting off the removal of cholesterol from the body. This leads to an increased amount of atherogenic particles in the blood circulation.

Sucrose is composed of glucose and fructose. The latter is highly lipogenic because it bypasses the key rate-limiting enzyme (phosphofructokinase) in the glycolytic pathway and results in the production of triglycerides **(39,40).** In response to the consumption of sucrose-rich diet (Z diet) the amount of the apoB-containing VLDL particles (triglyceride-rich VLDL) increases in C57BL/6J mice.

Saturated fat (T diet) has more neutral effects. There is a moderately increased plasma cholesterol, but not as severe as cholesterol-containing diets.

In vitro and *in vivo* studies have demonstrated that the transcriptional process controls the basal and induced expression of the three genes of fibrinogen **(41-44).** One of the intriguing observations in our study is, that despite a decrease in hepatic fibrinogen

Aα-, Bβ-, and γ-chain mRNA levels, the plasma fibrinogen levels significantly increased in all strains. To explore whether the acute phase response is involved in the increase observed in plasma fibrinogen levels, we measured two established acute phase markers. Our results demonstrated that these two markers respond to N diet, indicating that an acute phase response was present in this case. However, the fibrinogen mRNAs did not respond to this diet. Our findings are supported by Morlese *et al* **(45)** who have shown that four of the five positive acute phase proteins respond to infection but fibrinogen does not. Kamphuizen *et al* **(46)** have recently shown that the increase of plasma fibrinogen levels in patients with inflammation occurs independently of the acute phase reaction. These data suggest that the response of fibrinogen as an acute phase marker depends on the type of stimulation. The data from our study further indicate that regulation of the blood fibrinogen level is not determined by transcription alone, suggesting the involvement of post-transcription regulatory mechanisms such as translation and / or posttranslational modification, assembly of the fibrinogen from the chains, secretion, degradation **(47)**, and clearance.

Many epidemiological studies suggest a strong link between high plasma fibrinogen levels and an increase in the risk of cardiovascular disease. The current opinion is that the increase observed in plasma fibrinogen levels can be explained by atherosclerosis-induced acute phase response that leads to high fibrinogen mRNAs which in turn lead to high fibrinogen levels in plasma. On the basis of this hypothesis, fibrinogen is only considered as a marker of atherosclerosis and not as a causal factor involved in the development of atherosclerosis. Our results, however, demonstrate that diet-induced plasma fibrinogen levels can increase independently of mRNA levels.

It is intriguing to note that the strongest and most rapid increase of plasma fibrinogen is observed in C57BL/6J mice, which are known to be highly susceptible to diet-induced atherosclerosis **(27-30).** In the same strain and to a lower degree in BALB/C mice a strong and rapid increase of the acute phase marker haptoglobin was observed. Thus, the degree of acute phase response induction after consumption of an atherogenic diet is most pronounced in these mice. This is in agreement with the other observations showing that in response to the atherogenic (N) diet, C57BL/6J mice exhibited a dramatic induction of inflammatory genes, more aortic lesions, and a marked and rapid decrease in the level of HDL cholesterol and apolipoprotein A-I, whereas C3H/HeJ mice demonstrated almost no induction of inflammatory response, little or no atherosclerotic lesion development, and no changes in the HDL cholesterol

levels. Strain BALB/C mice were intermediate with respect to all above-mentioned factors **(27-30, 39, 40, 48, 49).** It has been suggested that differences between above-mentioned strains with respect to aortic lesion development, degree of inflammatory induction and plasma HDL levels and Apo A-I in response to an atherogenic (N) diet is determined by a major genetic factor, designed Ath-1 **(27-30, 39, 40, 48, 49).** This gene could be also involved in the determination of fibrinogen levels in plasma.
The observation that the diet-induced increase in fibrinogen was very fast in the highly atherosclerosis-susceptibile strain C57BL/6J and very slow in the atherosclerosis-resistant strain C3H/HeJ might point to the conclusion that it is the kinetics of the fibrinogen response and not the actual level which relates to atherosclerosis susceptibility.

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Chapter III

Increased hepatic fibrinogen Bβ-gene transcription is not enough to increase plasma fibrinogen levels. A transgenic mouse study.

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in press

ABSTRACT

The fibrinogen Aα, Bβ, and γ polypeptides are encoded by three separate genes, which are arranged in the order γ, Aα, Bβ. In order to study the biosynthesis of fibrinogen *in vivo* we generated a line of transgenic mice carrying extra copies of the fibrinogen Bβ-gene. To clone the mouse fibrinogen Bβ-chain gene, a mouse 129 Sv/Ev genomic cosmid library was screened, using the mouse fibrinogen Aα-, Bβ-chain cDNA. A clone containing the complete fibrinogen Bβ-chain gene including approximately 11-kb of the natural promoter region was identified and subsequently microinjected into mice. Southern blot analysis identified a founder that carried additional copies of the fibrinogen Bβ-chain gene. Transgenic offspring of this founder were interbred and heterozygous and homozygous transgenic mice were obtained. Northern blot analysis demonstrated approximately a 3-fold increase in fibrinogen Bβ mRNA in heterozygous mice as compared to wild-type, whereas homozygous transgenic mice showed approximately a 9-fold increase. The levels of the Aα and γ mRNAs in transgenic homozygous mice were not changed as compared to those in wild-type mice. Fibrinogen levels in plasma were not significantly increased in transgenic as compared to wild-type mice. These results indicate that: additional copies of the fibrinogen Bβ-chain gene lead to increased levels of the Bβ-chain mRNA in the liver; the increased levels of Bβ-chain mRNA in homozygous overexpression mice do not change the transcription levels of the two other fibrinogen mRNAs *in vivo*; the absence of an increased plasma fibrinogen level in the transgenic mice indicates that this level is not regulated solely by transcription of the Bβ-chain gene.

INTRODUCTION

Elevated plasma fibrinogen has been shown in numerous epidemiological studies to be associated with a greater risk of cardiovascular disease **(1-9)** and atherosclerosis **(10-13)**. Some studies suggest that a combined high level of fibrinogen together with a high level of serum cholesterol increases the risk of cardiovascular disease incidence to a greater extent than the other individual risk factors. Furthermore, the risk associated with hypercholesterolemia remains low when combined with low fibrinogen concentrations **(12).** Since the above-mentioned studies suggest that a high

level of plasma fibrinogen plays a role in the severity of atherosclerosis, it is important to gain more knowledge about the regulation of fibrinogen. We have therefore undertaken an *in vivo* study of fibrinogen biosynthesis in the mouse.
Fibrinogen is a plasma glycoprotein, synthesized by hepatocytes, composed of three different polypeptides: Aα , Bβ, γ **(14-16)**, encoded by three separate genes **(17-19).** The genes are arranged in the order of γ-Aα-Bβ, with the gene for Bβ-chain transcribed in the opposite direction **(20, 21).**
In vitro studies have suggested that overexpression of any fibrinogen chain mRNA leads to increased synthesis of the other two chain mRNAs resulting in a coordinated elevation in fibrinogen secretion **(22, 23).** However, transfection with Bβ cDNA had a more pronounced effect than did transfection with Aα or γ cDNA. It has been shown that there is an excess of Aα- and γ-chains in human hepatoma cells and that synthesis of the Bβ-chain is rate-limiting for the assembly and secretion of mature fibrinogen **(23-26).** Therefore, we directed our studies toward the fibrinogen Bβ-gene biosynthesis *in vivo*.
We generated transgenic mice with overexpression of the fibrinogen Bβ-gene and determined whether additional copies of the fibrinogen Bβ-gene lead to an increased plasma fibrinogen level.

MATERIALS AND METHODS

Preparation of construct, microinjection, and animals

As a first step to generate mouse fibrinogen Aα, Bβ and γ cDNA probes, a reverse transcription reaction was performed using 2 μg of total RNA isolated from mouse liver (C57BL/6J), according to the instructions of the kit (Gibco-BRL, Breda, The Netherlands). Since mouse fibrinogen Aα, Bβ, and γ cDNAs sequences were unknown, the primers used in polymerase chain reaction (PCR) amplification were derived from published rat fibrinogen Aα, Bβ, and γ cDNA sequences (Genbank accession numbers: X86561, U05675, and J00734 respectively) (table 1), (Isogen, Utrecht, The Netherlands). The PCR fragments were purified from the agarose gel using a gel extraction kit (Genomed-ITK, Uithoorn, The Netherlands) and subsequently cloned into a pCR II vector using a TA cloning kit (Invitrogen, De

Schelp, The Netherlands). The inserts were excised from the PCR II vector by digestion with *Eco*RI (Gibco-BRL, Breda, The Netherlands), and subsequently used as cDNA probes.

A mouse 129 Sv/Ev genomic cosmid library **(27)** was screened and rescreened with mouse fibrinogen Aα, Bβ, and γ cDNA probes. A clone containing the complete fibrinogen Bβ-chain gene including approximately 11-kb of the natural promoter region was identified. This cosmid also contained the intergenic region between Aα- and Bβ-chain gene and approximately the entire fibrinogen Aα-chain gene, ending in the middle of exon I of this gene. An approximately 34-kb insert was excised from this cosmid by digestion with *Not*I. The resulting fragment contained terminal T7, T3 standard primer sequences (table 1), and a *Bam*HI site, specific for this insert and not occurring in wild-type mice. Approximately 200 bp of both termini of the above-mentioned insert were sequenced with standard T7 and T3 primers (Base Clear, Leiden, The Netherlands), located at the end of exon I of fibrinogen Aα-chain gene and at the end of the fibrinogen Bβ-chain promoter (11-kb) of our insert respectively. On the basis of these sequences we derived two new primers T7R (T7 reverse) and T3R (T3 reverse), which were used in the PCR. Genomic DNA as a template in PCR with primer set T7 - T7R (Isogen, Utrecht, The Netherlands) generates an approximately 190-bp product whereas primer set T3 - T3R results in an approximately 160-bp amplification product.

The *Not*I fragment was purified and microinjected into fertilized mouse eggs taken from superovulated (C57BL/6J x CBA/J) F1 female mice purchased from Jackson laboratory (Bar Harbor, U.S.A). Heterozygous fibrinogen Bβ-chain overexpression

cDNA	Forward primer 5'-3'	Reverse primer 5'-3'
RAT Fbg Aα	CGTGGCCCAAGAATTGTGGAGAC	AGAGCGGATCTTGATATCAATGTC
RAT Fbg Bß	GCCCGTGGTCATCGACCTGTTGAC	CCAGACACCACCGGGATGTTGC
RAT Fbg γ	CTTACCAAACCGACGTGGACAC	GTCATTGTCCCAGGTACTGAAGTGC
DSP	Forward primer 5'-3'	Reverse primer 5'-3' (M)
T7	GTAATACGACTCACTATAGGGC	AAAATTAAGGTTAGGAGTAAC
T3	AATTAACCCTCACTAAAGGG	TTATCCTCTATCAGTGTAGG

Table 1. Primer pairs used for PCR reaction.
Abbreviations are fibrinogen (Fbg), mouse (M), complementary DNA (cDNA), Diagnostic set primers (DSP). The reverse primers used in Diagnostic PCR were derived from mouse sequence after sequences of the construct (for details see Materials and Methods, or figure 1).

mice (C57BL/6J X CBA/J F4 intercross) were crossed with C57BL/6J for 2 generations. Offspring from these crosses were intercrossed to yield the wild-type, heterozygous and homozygous mice of either gender used in this study.

Identification of transgenic mice

Transgenic mice were identified by either Southern blot hybridization (mouse fibrinogen Aα cDNA probe) or PCR (primers from table 1) on genomic DNA isolated from mouse tail biopsies by the proteinase K (Gibco BRL, Breda, The Netherlands) / sodium dodecyl sulfate (SDS) method **(28)**.

Collection of blood and tissue

Whole blood was collected into chilled tubes coated with EDTA (Sarstedt, Etten-Leur, The Netherlands) from the tail vein of mice. Plasma was prepared by centrifugation of whole blood at 3000 x g for 20 minutes at 4 °C and stored at -20 °C until analysis. Upon termination of the experiments, mice were sacrificed by cervical dislocation after which the livers, spleens, kidneys, lungs, and brains were removed and placed directly into liquid nitrogen and stored at -70 °C until analysis.

Northern blot analysis

Total RNA was isolated from liver, spleen, lung, brain and kidney tissues of transgenic and nontransgenic mice using the RNAzol™ B procedure (Cinna/Biotecx, Veenendaal, The Netherlands), fractionated by electrophoresis on a denaturing agarose gel, transferred capillarly to Nylon Hybond N^+ (Amersham-Pharmacia, Roosendaal, The Netherlands), UV cross-linked, and hybridized with [^{32}P]-labeled (Amersham-Pharmacia) probes of mouse fibrinogen Aα- Bβ- and γ-chain cDNAs, and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA **(29)**. The hybridization signal was quantified with a Phosphor-Imager (Fuji Fujix BAS 1000) and analyzed with the computer programs BAS-reader and TINA version 2.8 and 2.08c. The gel figures were based on a Phosphor-Imager file. The amounts of mRNA of fibrinogen were normalized to the levels of GAPDH mRNA.

Plasma fibrinogen analysis

Mouse fibrinogen concentrations were measured by sandwich ELISA, using purified polyclonal rabbit anti-rat fibrin antibodies **(30)** for coating and purified polyclonal goat anti-mouse fibrinogen antibodies conjugated to peroxidase as conjugate (Nordic, Tilburg, The Netherlands). The detection was performed by the immunoperoxidase procedure using tetramethylbenzidine (TMB) as a substrate (Organon Teknika, Boxtel, The Netherlands). The fibrinogen concentration of pooled citrated plasma from mice was determined by gravimetrical analysis **(31).** This pooled plasma was subsequently used as the standard in ELISA.

RESULTS

Generation of transgenic mice carrying extra copies of the mouse fibrinogen Bβ-chain gene

Mouse fibrinogen Bβ-chain gene with approximately 11-kb of the natural promoter region was used for microinjection. Figure 1 shows the construct that we used to generate transgenic animals. One out of the nine newborn mice was found to carry the transgenic sequences, as detected by Southern blot analysis, using a mouse fibrinogen Aα cDNA probe (figure 2). As depicted in figure 2, founder 4 showed an extra 4-kb *Bam*HI fragment of the genomic DNA as compared to wild-type mice. This extra fragment results from the extra *Bam*HI site, specific to our insert (at the end of exon I of the fibrinogen Aα-chain gene) and it did not occur in wild-type mice. These results were confirmed by PCR (data not shown).

To obtain homozygous mice carrying extra copies of the fibrinogen Bβ-chain gene, we intercrossed (C57BL/6J 75 % X CBA/J 25 %) for 3 generations. From the intensities (measured by Phosphor-imager analysis of the Southern blots) of the transgene specific 4.0-kb and wild type specific 7.0-kb fibrinogen bands the number of incorporated transgene copies were estimated. In heterozygous transgenic mice (figure 3, 6-11) the intensities of these bands were almost equal whereas in homozygous mice (figure 3, 12-17) the 4.0-kb band was approximately 3-4 times more intense than the 7.0-kb band. These data were best compatible with the presence of 3 extra copies of the transgene in the heterozygous mice and 6 extra copies in the homozygous mice. This brings the total number of Bβ-gene copies to 5 en 8

respectively. The homozygosity for the transgene was confirmed by mating one of the predicted homozygotes (male) with nontransgenic (female) mice, resulting in offspring which were all positive for the integrated gene.

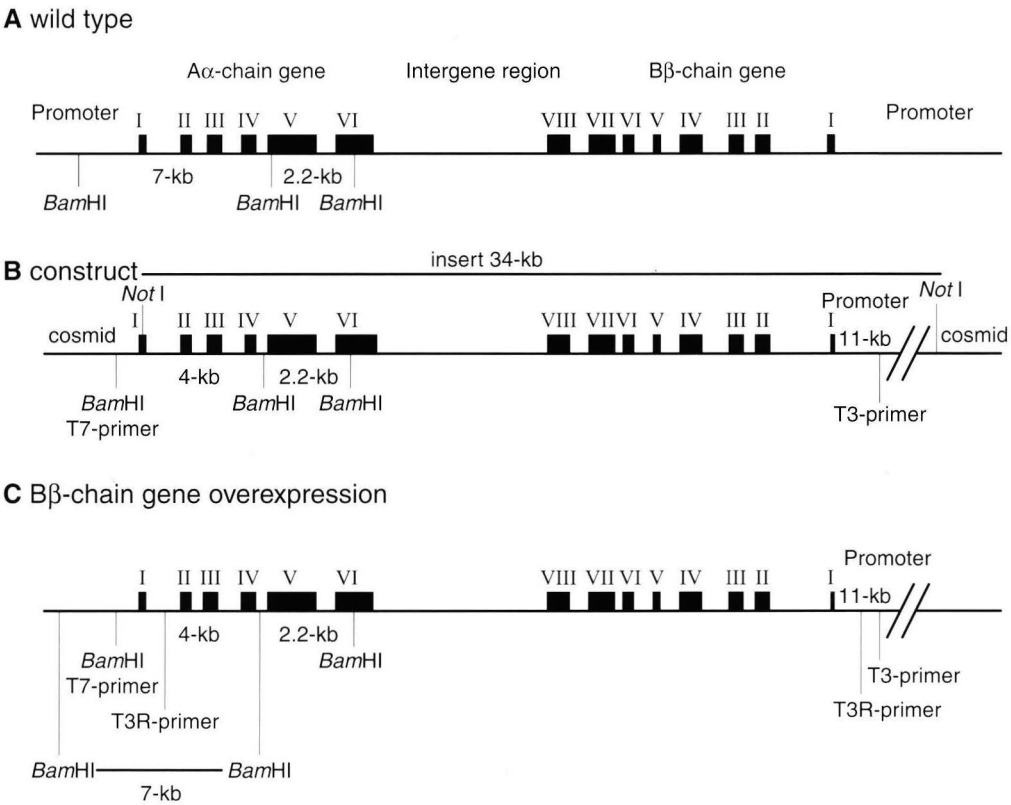

Figure 1. Schematic representation of the mouse fibrinogen Bβ-chain overexpression construct.
The exons are numbered and indicated by solid black boxes. Sizes of relevant restriction fragments, insert, and the promoter of Bβ-chain gene are given in kilobases. (**A**) Structure of the normal mouse fibrinogen Aα- and Bβ-chain genes. (**B**) The DNA construct used for microinjection. The 34-kb DNA insert was isolated from the cosmid vector by digestion with *Not*I. This insert contains at the 5'-end an extra *Bam*HI site and T7-forward sequences and at the 3'-end T3-forward sequences. This insert was used for microinjection. (**C**) The structure of mouse fibrinogen Bβ-chain gene overexpression construct. The reverse primers for T7-forward and T3-forward primers are shown by T7R and T3R. The combinations of T3F with T3R and T7F with T7R were used in PCR.

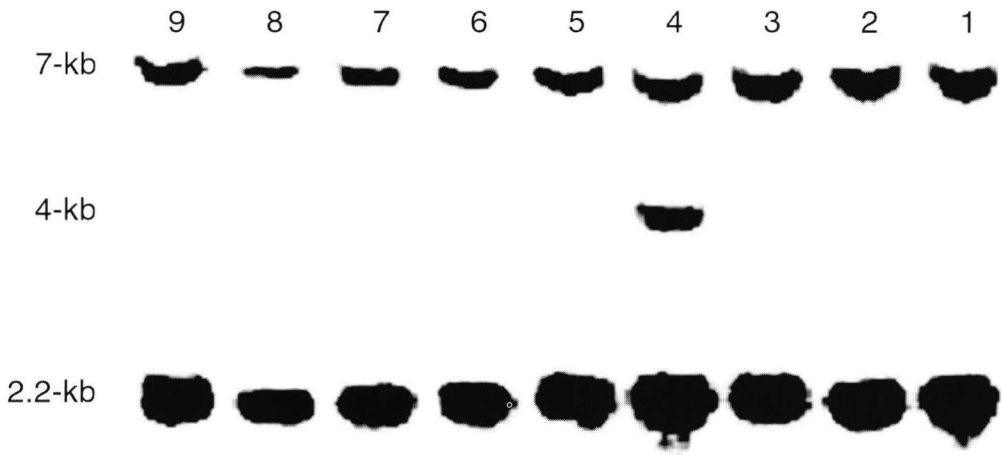

Figure 2. Identification of founder mice carrying transgenic sequences.
Southern blot hybridization of genomic DNA of 9 newborn mice after *Bam* HI digestion with fibrinogen cDNA Aα-probe. The samples are numbered from 1 to 9. The sizes of the *Bam*HI fragments are given in kilobases. The approximately 7-kb and 2.2-kb *Bam*HI fragments were obtained in every mouse. The approximately 4-kb *Bam* HI fragment was only obtained in mouse 4.

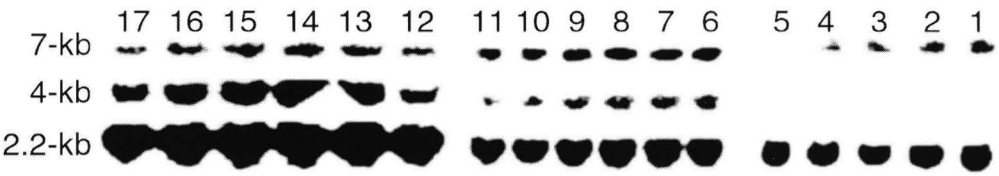

Figure 3. Identification of homozygous and heterozygous transgenic offspring.
Southern blot hybridization of genomic DNA of transgenic offspring after *Bam*HI digestion with fibrinogen cDNA Aα-probe. Comparison of the intensity of the 4-kb *Bam*HI fragment with the approximately 7-kb fragment intensity was used to discriminate between heterozygous and homozygous transgenic mice. The samples are numbered from 1 to 17. The size of *Bam*HI fragments is indicated in kilobases. 1-3 female, 4-5 male wild-type mice; 6-8 female, 9-11 male heterozygous transgenes; 12-14 female, 15-17 male homozygous transgenes.

Expression of fibrinogen mRNAs

To determine whether the number of fibrinogen Bβ-chain gene copies in mice affects the level of hepatic fibrinogen Bβ-chain mRNA expression, we measured the fibrinogen mRNA level in the liver. Northern blot analysis showed that the level of fibrinogen Bβ-chain mRNA in heterozygous mice is approximately three-fold higher than in non-transgenic littermates (table 2, and figure 4). Furthermore, the expression of fibrinogen Bβ-chain mRNA was approximately 9-fold higher in homozygous mice

than in control mice (figure 4). We observed no differences between transgenic and non-transgenic mice with respect to fibrinogen Aα and γ mRNA expression (in some cases significant but absolutely not comparable with the dramatic increase of Bβ-mRNA levels, see table 2).

Genotype	γ (Pls-bkg = 100%)	Aα (Pls-bkg = 100%)	Bβ (Pls-bkg = 100%)	mg/ml
Wild type				
F	100 ± 2	100 ± 7	100 ± 10	1.36 ± 0.09
M	100 ± 13	100 ± 13	100 ± 21	1.76 ± 0.09
Homozygotes				
F	140 ± 18 *	126 ± 5 *	815 ± 3 *	1.45 ± 0.06
M	110 ± 10	129 ± 20	970 ± 7 *	1.94 ± 0.05

Table 2. Hepatic fibrinogen mRNA and plasma fibrinogen levels in wild-type and transgenic mice.
Fibrinogen Aα, Bβ, and γ mRNAs (Aα, Bβ, and γ respectively) were determined in the liver of wild-type, heterozygous (not shown) and homozygous (overexpression) mice by Northern blot. Mouse fibrinogen mRNA levels in liver are relative to an internal standard GAPDH and are expressed as a percentage of wild-type mice. Values for all measurements are expressed as the mean ± SD of 3 mice per group. The fibrinogen concentrations were determined in the plasma of non-transgenic and transgenic mice of either gender by an immunological method (ELISA). Mouse fibrinogen levels in plasma are expressed in mg/ml (mean ± SD of 4-6 mice per group). $P < 0.05$ was accepted as statistically significant (t-test) and indicated by an asterisk. The abbreviations in the table are pulse minus background (pls-bkg), female (F), and male (M). For details see Materials and Methods.

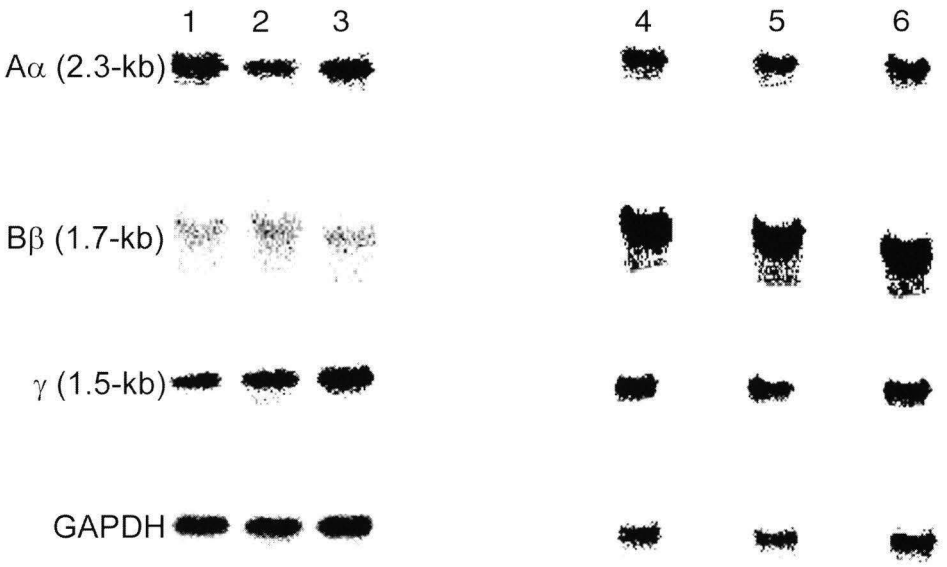

Figure 4. Northern blot hybridization analysis of fibrinogen mRNA levels in wild-type and transgenic mice.
Fibrinogen γ, Aα, and Bβ mRNAs were determined in the liver of wild-type and homozygous (overexpression) mice by Northern blot. Three male mice per group were used. The lanes 1-3 and 4-6 represent wild-type and homozygous mice respectively. For details see also the legend of table 2 and Materials and Methods.

To explore whether the expression of all three mRNAs of fibrinogen is restricted to liver tissue, we also analyzed fibrinogen mRNAs in spleen, lung, brain and kidney tissues. With Northern blot analysis, the three fibrinogen chain mRNAs could not be detected in those tissues (data not shown).

Plasma fibrinogen analysis

To explore whether the increased hepatic fibrinogen Bβ-chain mRNA levels lead to the increase of plasma fibrinogen levels in transgenic mice, we measured the fibrinogen concentrations in the plasma of either wild-type or transgenic mice. We observed no significant differences in plasma fibrinogen levels between wild-type mice and heterozygous or homozygous transgenic mice of either gender (table 2). However, significant differences were observed between female and male mice with respect to plasma fibrinogen levels.

Discussion

Northern blot analysis showed that the expression of fibrinogen Bβ-chain mRNA was higher in heterozygous and homozygous transgenic mice of either gender than in wild-type mice. Quantitative analysis demonstrated an approximately 2-3-fold increase in copy number of the fibrinogen Bβ-gene in heterozygous and a 3-4-fold increase in homozygous mice resulting in an approximately 3- and 9-fold increase of the fibrinogen Bβ-chain mRNA levels respectively. These results indicate that the increase in the level of fibrinogen Bβ-chain mRNA in transgenic mice is higher than expected from a linear relationship between gene copy number and mRNA levels. The Bβ-chain gene overexpression construct was derived from a mouse 129 Sv/Ev genomic cosmid library and subsequently microinjected into C57BL/6J mice. In a previous study **(32)** we showed that the 129 Sv/Ev mouse strain exhibited significantly higher plasma fibrinogen levels than in C57BL/6J mice. This could indicate a higher transcriptional activity of the 129 Sv/Ev fibrinogen gene or increased stability of the 129 Sv/Ev fibrinogen Bβ-chain mRNA. Another explanation for the higher than expected fibrinogen Bβ-chain mRNA levels could be related to the sites of integration of the transgene (fibrinogen Bβ-chain gene), which could allow for the higher transcription activity of fibrinogen Bβ-chain transgenes as compared to the endogenous gene. To determine whether the additional copies of the fibrinogen

Bβ-chain mRNA observed in the transgenic mice lead to the increase of plasma fibrinogen level, we measured fibrinogen in the plasma of transgenic and non-transgenic littermates. Surprisingly, no increase in plasma fibrinogen level was observed, indicating that higher fibrinogen Bβ-chain mRNA levels do not lead to an increased plasma fibrinogen level. These results suggest that the plasma fibrinogen level is not determined by transcription of the fibrinogen Bβ-chain mRNA alone. Note that if the synthesis of Bβ-chain is the rate-limiting step in the assembly and secretion of fibrinogen, we would expect an increase of plasma fibrinogen in transgenic mice. A mouse fibrinogen Aα-chain knock-out study suggests that the level of Aα-chain is also not a rate-limiting factor in fibrinogen production **(33)**. However, overexpression of all three fibrinogen genes has been shown to lead to increased plasma fibrinogen levels in mice **(34),** pointing to a required co-ordinate overexpression of all three chains. This suggests the involvement of transcription regulation of other fibrinogen genes **(26, 34, 35),** post-transcription regulatory mechanisms, such as translation, and/or post-translational modification, assembly, secretion and degradation **(36)**, or interplay between these processes.

No differences were observed between homozygous transgenic mice and wild-type mice with regard to fibrinogen Aα-, and γ-chain mRNA expression. These results indicate that the overexpression of Bβ-chain of fibrinogen does not alter the transcription of the other two endogenous fibrinogen genes**.** The same mouse fibrinogen Aα-chain knock-out study **(33)** resulted in a similar conclusion. In this study it was shown that fibrinogen Aα-chain mRNA was not detectable in the liver of homozygous knock-out mice, whereas fibrinogen Bβ-, and γ- chain mRNA levels were comparable in the same mice.

These *in vivo* findings are in contrast to *ex vivo* studies (using HepG2 cell lines) that have shown the Bβ-chain **(23-26)** or Aα-chain **(37)** to be the rate-limiting step in the assembly and secretion of fibrinogen. The observed variation in *ex vivo* studies could be due to variations in the origin of cell lines and culture conditions. Furthermore, the effect of physiological parameters or other mechanisms may be missed in *in vitro* studies, while this is not the case in *in vivo* studies.

In conclusion, additional copies of the fibrinogen Bβ-chain gene lead to increased levels of the Bβ-chain mRNA in the liver, but the increased hepatic fibrinogen Bβ-chain mRNA levels do not result in elevated plasma fibrinogen levels, indicating that this level is not regulated solely by transcription of the Bβ-chain gene. The increased levels of Bβ-chain mRNA in homozygous overexpression mice do not affect the

transcription levels of the two other fibrinogen mRNAs *in vivo*. Furthermore, no evidence was found for a mechanism that coordinates the expression of the three fibrinogen genes.

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Chapter IV

A novel transgenic mouse model of hyperfibrinogenemia.

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in press

ABSTRACT

Hyperfibrinogenemia is a risk predictor in several diseases, including cardiovascular disease. Nevertheless, it remains unknown whether elevated fibrinogen has an etiologic role in disease pathogenesis or is a reflection of it, or both. To examine this question, we generated a mouse model of hyperfibrinogenemia. We isolated the mouse fibrinogen locus, containing the three fibrinogen genes, in a single P1 clone. This ~ 100-kb clone was injected into C57Bl/6J zygotes. Three transgenic lines were identified, two with elevated fibrinogen, 1.4- and 1.7-fold relative to normal. We characterized the line with the highest level. Northern blots of total RNA showed transgene expression was liver-specific, and the message levels were 2- to 3-fold enhanced. Fibrinogen in transgenic mice was normal in both immunologic and clotting assays. Our data indicate that over-expression of all three fibrinogen genes is necessary to achieve hyperfibrinogenemia. We saw no increase in mortality or morbidity, no gross abnormalities in the organs, and no histologic differences in lung, liver, spleen or kidney, in transgenic mice relative to normal littermates. We conclude that elevated fibrinogen did not cause disease in mice. We anticipate that breeding these mice to other mouse models of disease will demonstrate whether hyperfibrinogenemia has a role in the initiation or progression of symptomatic disease.

Key words: Hyperfibrinogenemia, mouse models, fibrinogen

INTRODUCTION

Fibrinogen is a plasma protein with primary functions in coagulation and wound healing. An abnormally high concentration of plasma fibrinogen, or hyperfibrinogenemia, is correlated with several diseases. Hyperfibrinogenemia is an independent risk predictor for ischemic events, usually associated with atherosclerosis **(1-6).** Elevated plasma fibrinogen is common in diabetics, and is a risk predictor for vascular complications in these patients **(7, 8).** Hyperfibrinogenemia is a component of the metabolic syndrome, a concurrence of at least two of four risk factors - the highest quartile for blood glucose, plasma triglycerides or blood pressure, or lowest quartile for plasma HDL **(9).** Patients with the nephrotic syndrome have an increased absolute rate of fibrinogen synthesis, 31 mg/kg/day compared to healthy

controls who synthesize fibrinogen at a rate of 21 mg/kg/day **(10).** Hyperfibrinogenemia is also associated with most solid carcinomas, with lung cancer showing the strongest correlation in 62 % of patients tested **(11).**

Fibrinogen is a 340 kDa glycoprotein composed of two copies of each of three polypeptide chains, Aα, Bβ, and γ. A separate gene encodes each polypeptide. As depicted in Figure 1 A, the three genes are found in a 50-kb locus composed of the γ-chain gene, ~ 15-kb of intervening DNA, the Aα-chain gene, another ~ 15-kb of intervening DNA, and the Bβ-chain gene **(12, 13).** The γ-chain and Aα-chain genes are transcribed from one strand while the Bβ-chain gene is transcribed from the opposite strand **(13).** Each coding region has its own promoter **(14-17),** so the boundaries of the locus are defined by the upstream elements that control transcription of the γ- and Bβ-chain genes. The genes are expressed constitutively in hepatocytes, where the three fibrinogen transcripts represent about 3 % of the total liver mRNA **(18).** Fibrinogen is also an acute phase protein, with increased gene expression mediated predominantly by interleukin-6 **(19, 20).** Thus, plasma fibrinogen levels are increased in response to inflammatory stress.

The correlation of hyperfibrinogenemia with disease may therefore reflect an association between disease and inflammatory stress, rather than implicating an etiologic role for hyperfibrinogenemia in disease. As a first step to provide an experimental means to distinguish these possibilities, we have generated a mouse model of hyperfibrinogenemia. We chose a transgenic approach, inserting a genomic segment with the complete mouse fibrinogen locus. We isolated a P1 phage containing the mouse fibrinogen locus, and introduced this locus into the mouse. We identified two founder mice with hyperfibrinogenemia, one with fibrinogen levels increased almost two-fold. In the future, combining this mouse model with models of atherosclerosis, renal disease, diabetes or cancer will provide a means to determine whether elevated fibrinogen has an etiology in the development of these diseases.

MATERIALS AND METHODS

All chemicals were reagent grade from Sigma (St. Louis, MO), unless noted. Restriction enzymes were from New England BioLabs (Beverly, MA). Supermix and agarose for PCR were purchased from Life Technologies (Rockville, MD). C57Bl/6J mice for microinjection were purchased from Jackson Labs (Bar Harbor, ME); C57Bl/6J mice for breeding were purchased from Taconic (Germantown, NY).

Polyclonal antibodies were: rabbit anti-human fibrinogen from Dako (Carpinteria, CA); rabbit anti-human γ-chain prepared by Hazelton Research Products (Denver, PA) using γ-chain purified from inclusion bodies expressed in *E. coli* as the antigen **(21),** rabbit anti-human α-chain, a gift from Dr. Edward Plow (Cleveland Clinic); peroxidase-conjugated goat anti-mouse fibrinogen from Nordic Immunologica (Boxtel, The Netherlands); peroxidase-conjugated goat anti-rabbit IgG from Calbiochem (La Jolla, CA). Hybond N^+ membrane, ECL Western blotting detection reagents, Megaprime kit for primers and radio-labeled ^{32}P was purchased from Pharmacia-Amersham (Piscataway, NJ/ Roosendaal, The Netherlands). Agarose for the Northern and Southern blots was from Boehringer Mannheim (Almere, The Netherlands).

Isolation of mouse fibrinogen locus

Genome Systems (St. Louis, MO) screened a mouse 129 Sv/Ev P1 library, using primers (forward 5'-CCTGATGGTCGCCGAGAAAT and reverse 5'-CTACTGGAAGAAAACTCAGG) from mouse Aα-chain cDNA (pair 1 in Figure 1 A; sequence kindly provide by J. Degen), and provided four positive clones.
Clones with the entire locus were identified by PCR with primers from the γ-chain (forward 5'-GAAAACAGAACCACAGAAG and reverse 5'-GTGGCACCCTCTATCATA; pair 2 in Figure 1 A) and the Bβ-chain (forward 5'-TCAGCCAACAAGTGAACC and reverse 5'-ATAGATGCCTCATGGTTT; pair 3 in Figure 1 A) with 35 cycles of 94 ^{0}C 1 min, 50 ^{0}C 1 min, 72 ^{0}C 30 sec, and a final 4 min extension.

Generation of transgenic animals

Clone P1-35, containing the entire fibrinogen locus, was linearized with *Sac* II, isolated on a TAE-agarose gel (0.4 %) and purified by elutrap into injection buffer (10 mM Tris pH 7.4, 0.1 mM EDTA). The purified transgene (~20 ng/ml) was microinjected into the pronucleus C57Bl/6J zygotes; transgenic procedures were performed by K. Mohr at the UNC Animal Models Core Facility. DNA for PCR and Southern blotting was isolated from tail biopsies using a standard proteinase K/ phenol-chloroform extraction **(22).**

Fibrinogen Transgene (FT)-positive mice were identified by PCR using a forward primer from the Sp6 promoter in the vector (5'-GGCCGTCGACATTTAGGTGACAC) and a reverse primer from a sequence upstream of the γ-chain (5'-AGTCAATTTGGTCACTAACCGCC; primers FT assay in Figure 1 B), with 35 cycles of 30 sec at 94 °C, 55 °C and 72 °C.
Mice were housed in a full-barrier facility in autoclaved cages and were given unlimited access to water and food (Picolab20 Irradiated Mouse Diet breeder chow, Purina-Mills, St. Louis, MO). All procedures were approved by the Institutional Approval of Care and Use Committee.

Genomic analysis of FT+ insertion

Transgene insertion was confirmed and transgene copy number was estimated by Southern blot. Genomic DNA was digested with *Bam*HI, and 17 mg run in a 0.8 % agarose gel overnight. Blots were prepared and hybridized with a ^{32}P-labeled γ-chain cDNA as previously described **(23).** This probe hybridized with two bands in FT+ mice, an 8-kb band, present only in the FT+ mice, and a 12-kb band present in all mice. Transgene copy number was estimated by densitometric comparisons of these two bands. Tandem insertions of the transgene were identified by PCR using the primers 'head' (5'-TAAAACCCGCAACCCTAC) and 'tail' (5'-ACGCCTTCCTGAAAAATG) shown in Figure 2 A with 35 cycles of 94 °C 1 min, 55 °C 30 sec and 72 °C 1 min.

Northern blot analysis

Total RNA was isolated from the livers, lungs, testes, brains, and kidneys of 3 FT+ mice, with the RNAzol™ B procedure (Teltest, Inc, Veenendaal, The Netherlands). RNA (10 mg) was separated on a 1.2 % denaturing agarose gel containing 0.75 % formaldehyde (Merck, Amsterdam). The gel was transferred to Nylon Hybond N$^+$, UV cross-linked and hybridized with ^{32}P-labeled cDNAs for each chain, according to Megaprime kit instructions. Quantitative Northern blots were prepared using total RNA from the livers of 3 FT+ and 3 FT- mice; a ^{32}P-labeled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as a loading control **(24).** Bands

were quantified with a Fuji Fujix BAS 1000, normalized with GAPDH values, and analyzed with the programs TINA v. 2.09g (Raytest Isotopen Messgeraete, GmbH, Straubenhard, Germany).

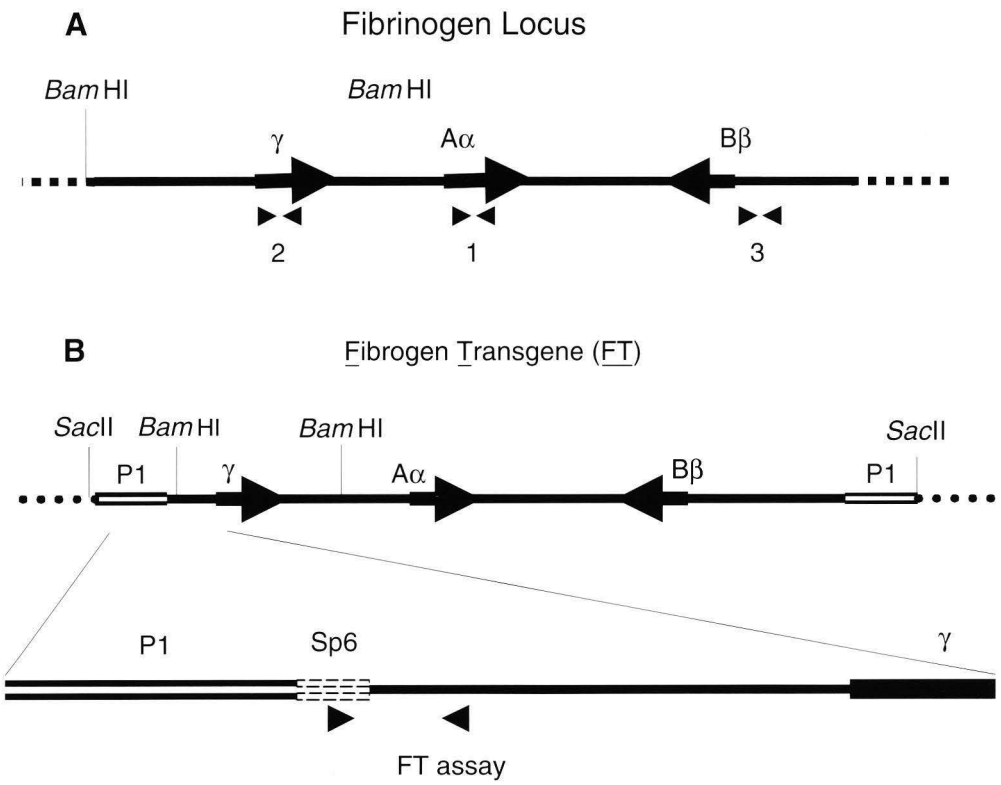

Figure 1. The fibrinogen locus and fibrinogen transgene derived from clone P1-35.
In both figures, each individual gene is represented as an arrow pointing from 5' to 3'. **A**. Endogenous mouse fibrinogen locus based on human and rat data including the coding regions for γ, Aα and Bβ chains. PCR primer pairs (1, 2 and 3) used for selection of the P1 clone and characterization of the insert are marked. Selected *Bam* HI sites are shown. **B**. Map of P1-35, linearized at the *Sac* II site to create the Fibrinogen Transgene (FT). PCR primer pairs for genotyping (FT assay) are shown in the close up of the transgene; one primer binds to the Sp6 site within the P1 vector and the other binds 5' to the γ-chain within the fibrinogen locus. Selected *Bam* HI sites are shown.

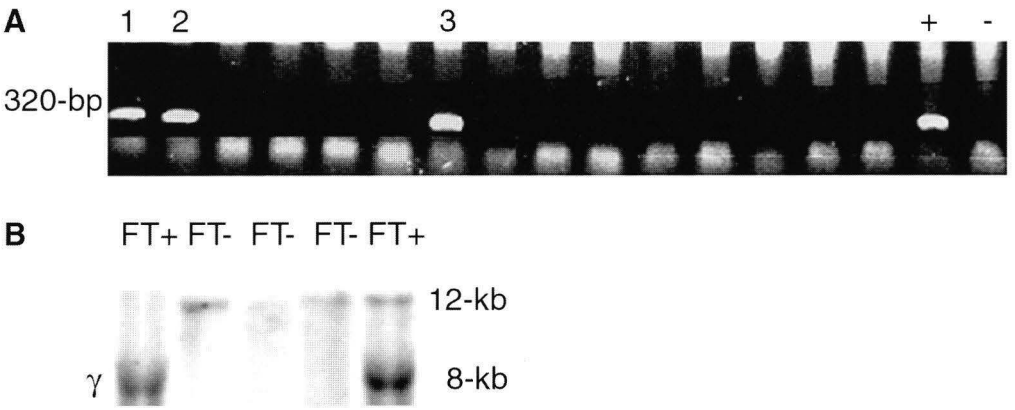

Figure 2. Detection of the Fibrinogen Transgene (FT).
A. FT assay PCR was performed on genomic DNA from 15 microinjected mice. Positive control (P1-35) produced the predicted band of 320 bp in lane +. Negative control (C57Bl/6J genomic DNA) did not produce a band in lane -. Mice 1, 2 and 3 were positive for the transgene (FT+) whereas the other 12 mice were negative. **B**. Southern blot analysis was performed using genomic DNA from animals identified as positive or negative by the FT assay PCR. Blot was hybridized with a γ-chain probe. *Bam*HI digests produced a 12-kb band in all animals from the endogenous locus (see Figure 1 A) and an 8-kb band from the transgene in FT+ animals (see Figure 1B).

Collection of blood

Blood was drawn from mice anesthetized with ketamine (Barber Veterinary, Fayetteville, NC) into heparinized capillary tubes (Fisher, Suwanee, GA) inserted into the retro-orbital space. Samples (~50 µl) were transferred to microfuge tubes and spun at 15,000 rpm for 15 minutes at ambient temperature; plasma was stored at -80 ^{0}C.

Plasma fibrinogen analysis

A mouse-specific fibrinogen ELISA was developed from standard protocols **(25)**, J. Koopman, personal communication). Plates (Immulon 4-96 well, Fisher Suwanee, GA) were coated with a 1:1000 dilution of anti-human fibrinogen antisera, and washed with PBS containing 0.05 % Tween 20 (PBST); there was no blocking step. Plasma samples were diluted 1:10,000 in PBST, followed by two half-step dilutions, each plated in triplicate. A mouse plasma standard was prepared from pooled blood obtained by heart puncture from 11 C57Bl/6J X CH3/HeJ F1 mice. The plasma standard was diluted 1:400 into PBST, and serial ten two-fold dilutions used for a

standard curve. Plates were incubated for 2 hours at ambient temperature or overnight at 4 ^{0}C, washed and incubated with peroxidase-conjugated anti-mouse fibrinogen antisera (1:50000 in PBST) for 45 minutes at ambient temperature. TMB Microwell chromogenic substrate (3,3',5,5'-tetramethylbenzidine; KPL, Gaithersburg, MD) was added for 30 minutes, the reaction stopped with 100 ml of 1M phosphoric acid, and the absorbance read at 450 nm.

The pooled plasma standard and three samples from FT+ mice were tested by the UNC Hospitals Core Laboratories, using an Organon Teknika MDA 180 (Boxtel, The Netherlands) according to the manufacturer's directions. This method is based on a modified Clauss assay using human thrombin. Clotting times are based on values generated by human thrombin with human fibrinogen.

Western blot analysis

SDS-PAGE was performed according to Laemmli **(26).** Mouse plasma was diluted 1:5 in loading buffer with DTT, and ~100 ng of plasma fibrinogen or purified human fibrinogen (Calbiochem, La Jolla, CA) were analyzed on a 7.5 % acrylamide gel. Gels were transferred to nitrocellulose (Micron Separations, Westborough, MA) for 6 hours at 120 mA. The membranes were blocked overnight, in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.05 % Tween 20), with 5 % powdered milk overnight, incubated with anti-human fibrinogen antibody diluted 1:6000, or anti-human α- or γ-chain diluted 1:2000 in TBST with 1 % BSA for 2 hours at room temperature, and developed with alkaline phosphatase conjugated goat anti-rabbit antisera and ECL detection reagents.

Statistics

Statistical analyses were prepared on StatView for Windows (Abacus Concepts) using two sample t-tests.

RESULTS

Cloning of the mouse fibrinogen locus

Because the three fibrinogen genes are found in a 50-kb locus in humans **(13),** it seemed reasonable to look for a single P1 clone with an 80-100-kb insert containing the complete mouse locus, schematically shown in Figure 1 A. We designed a PCR screen with primers from the mouse Aα-chain cDNA (Figure 1 A, primer pair 1) for Genome Systems to screen their mouse (129 Sv/Ev) genomic P1 library. They identified four positive clones, which we analyzed by two additional PCR assays. Based on the known human and rat genomic sequences, we designed primers to amplify segments at the 5' ends of Bβ and γ genes (Figure 1 A, primer pairs 2 and 3). Two clones, P1-35 and P1-107, were positive for Bβ- and γ-chains, while each of the remaining clones was positive for only one of the primer pairs. Restriction enzyme mapping and sequence analysis of P1-35 and P1-107 gave the genomic arrangement in Figure 1 A and demonstrated that P1-107 contained only 300 bp 5' to the γ-chain mRNA start site. In P1-35, shown in Figure 1 B, the locus was approximately centered in the genomic insert, with at least 5-kb of sequence 5' to the γ-chain gene and at least 15-kb of sequence 5' to the Bβ-chain gene. As this clone contains all the known fibrinogen transcriptional regulatory elements **(14-17),** we used P1-35 as to generate transgenic mice.

Generation and identification of transgenic mice

P1-35 DNA was linearized with *Sac*II, purified by elutrap, and microinjected into C57Bl/6J zygotes, as described in methods. Approximately 40 pups were born, and fifteen survived until weaning. We identified Fibrinogen Transgene positive (FT+) pups with a PCR assay using one primer from the Sp6 site in the vector and one primer from the genomic locus about ~ 5-kb upstream of the γ chain (Figure 1 B, FT assay). As shown in Figure 2 A, 3 of the 15 pups were positive, demonstrating that at least this part of the transgene was present in each animal. Southern blot analysis of *Bam* HI digested DNA from line 1 hybridized to a γ-chain cDNA probe confirmed the transgene insertion. As shown in Figure 2 B, two bands were detected, an 8-kb band from the transgene, which has a *Bam* HI site introduced with the P1 vector, and a 12-

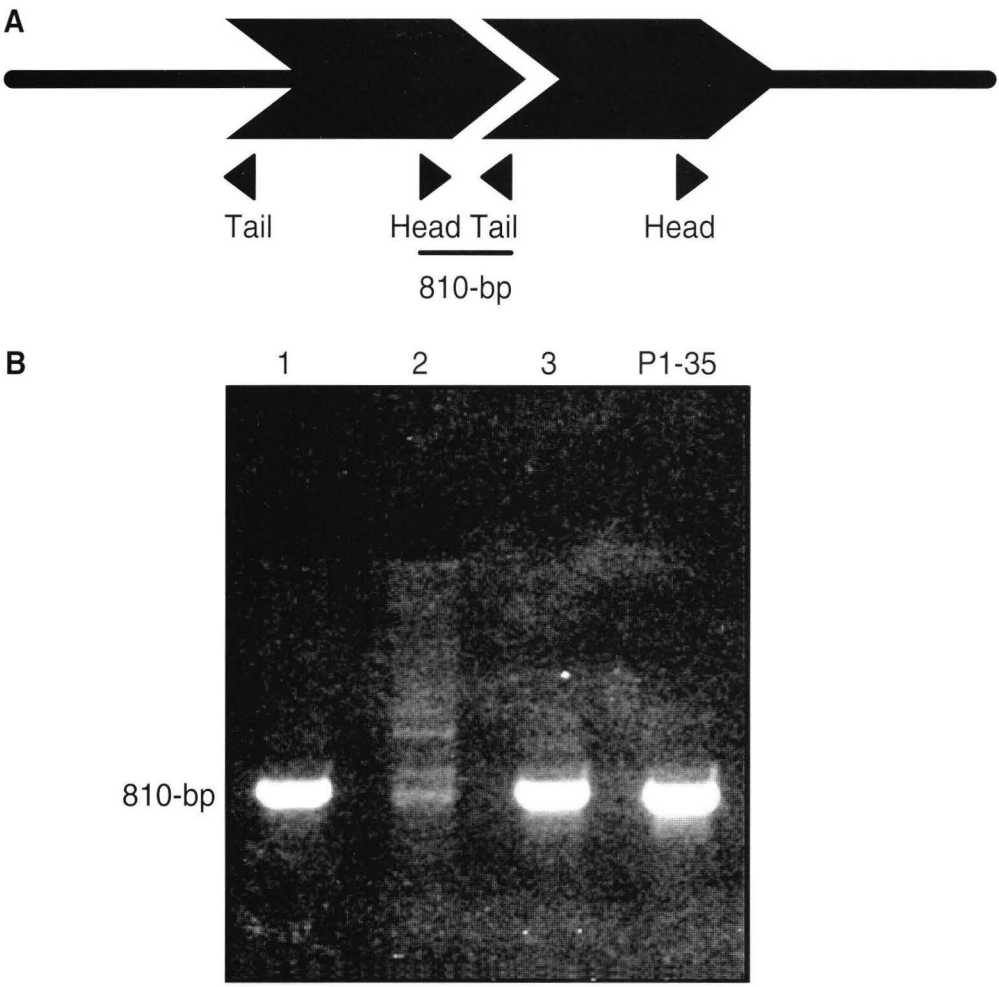

Figure 3. Analysis of tandem transgene insertion.
A. 'Head-to-Tail' PCR: primers were designed to generate an amplicon of 810 bp only if two (or more) copies of the transgene were inserted in tandem. **B.** 'Head-to-Tail' PCR analysis of lines 1, 2 and 3. The positive control (circular P1-35) and lines 1 and 3 gave a positive band of 810 bp. Line 2 did not show a positive band.

kb from the endogenous locus (see Figure 1 A). A comparison of the intensities of these two bands was used to estimate the number of inserted transgenes at 2-6 copies (data not shown).

We used a 'head-to-tail' PCR assay, shown in Figure 3 A, to determine whether multiple tandem copies of the transgene were present. As shown in Figure 3 B, lines 1 and 3 had tandem copies, indicating that the entire P1 transgene was present in these lines. No PCR product was detected with line 2, consistent with the insertion of a single copy of the transgene. Breeding the three founder mice, all female, to C57Bl/6J males demonstrated normal Mendelian inheritance of the transgene.

Fibrinogen levels in transgenic mice

Transgene expression was determined by measuring the fibrinogen concentration in plasmas from the first litter of each line. We developed a mouse-specific ELISA to determine total fibrin-related antigens, as described in methods. The capture antibody was a polyclonal raised against human fibrinogen and the peroxidase conjugated detection antibody was a polyclonal raised against mouse fibrinogen. Pooled mouse plasma was used as the standard. We found that FT+ mice from line 1 had nearly double (2.0 ± 0.1 mg/ml) the concentration of plasma fibrinogen when compared to FT- littermates (1.2 ± 0.1 mg/ml; p=0.0001). FT+ mice from line 2 also had elevated plasma fibrinogen relative to FT- littermates (1.7 ± 0.3 mg/ml and 1.2 ± 0.5 mg/ml, respectively; p=0.0010). FT+ mice from line 3 had fibrinogen levels that were not significantly different from FT- littermates (1.2 ± 0.1 mg/ml and 1.2 ± 0.1 mg/ml, respectively; p=0.9316). Subsequent analysis of litters from the founder containing 22 FT+ mice and 23 FT- mice confirmed the two-fold increase, with 2.0 ± 0.3 mg/ml for FT+, and 1.2 ± 0.5 mg/ml for FT-; $p < 0.0001$. After ELISA analysis of approximately 100 mice of each genotype from line 1, generated from the founder's offspring, FT+ mice have an average plasma fibrinogen of 2.0 ± 0.3 mg/ml as compared to 1.2 ± 0.2 mg/ml plasma fibrinogen in FT- mice. We concluded that FT+ mice of line 1 had multiple tandem insertions of the transgene with a significant increase in plasma fibrinogen. Thus, we focussed our further studies on this line.

Expression of fibrinogen mRNAs

We examined the tissue-specific expression from this genomic transgene by Northern blot analysis. Total RNA was prepared from liver, brain, testes, kidney and lung of an FT+ male mouse, and mouse cDNA probes for each of the three chains were used, as described in methods. As shown in Figure 4 A, fibrinogen expression was detected

only in liver RNA, indicating that expression from the transgene had the same tissue specificity as expression from the endogenous locus. We also measured the level of fibrinogen expression by Northern blot analysis with total RNA from livers from three FT+ mice and three FT- mice. The blot was developed successively with a mixture of Aα- and γ-chain cDNA probes, a Bβ-chain cDNA probe, and a rat GAPDH cDNA probe, which served as a loading control. These blots, shown in Figure 4 B, were quantified by densitometry and the average of the three FT+ and FT- mice, adjusted to the GAPDH controls, determined. The results, presented in Table 1, showed that steady state levels of fibrinogen mRNA in the liver of FT+ mice was 2.0-3.4 fold greater than in FT- mice.

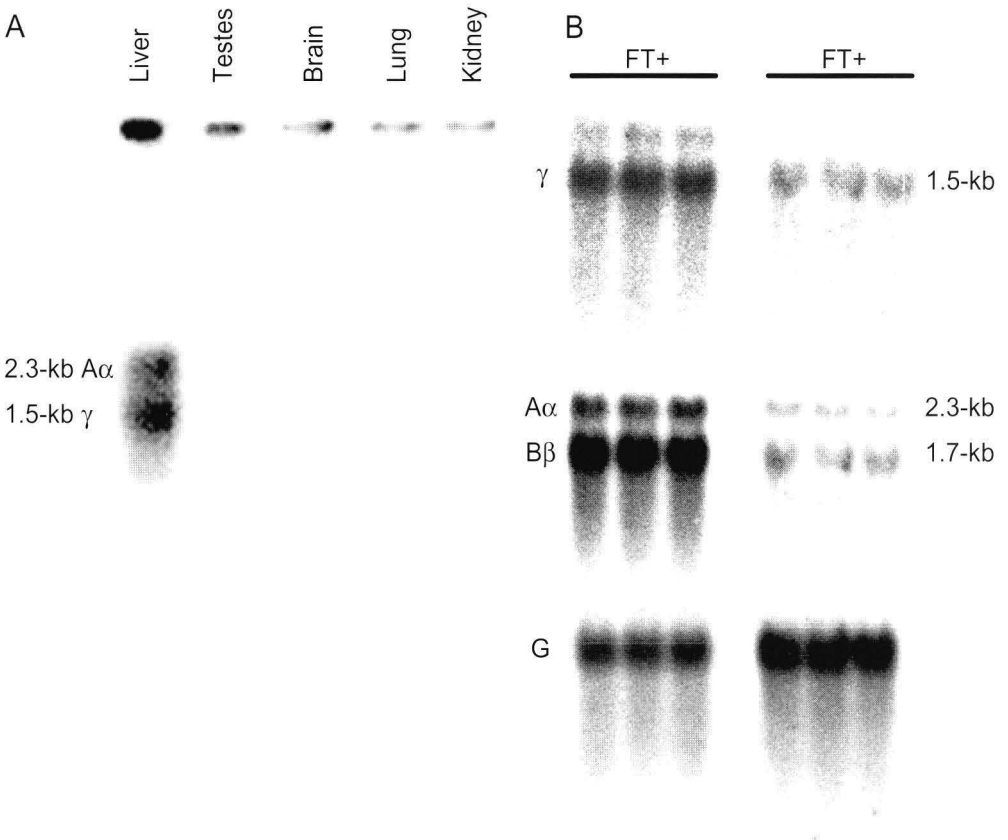

Figure 4. Analysis of fibrinogen RNA in FT+ and FT- animals.
A. Northern blot for tissue specificity. RNA was prepared from liver, testes, brain, lung and kidney of FT+ mice. Probes were for Aα (2.3-kb) and γ (1.5-kb); Bβ is not shown. **B**. Quantitative northern blot using liver RNA from FT+ and FT- mice. Aα-band is 2.3-kb; γ-band is 1.5-kb; Bβ-band is 1.7-kb. Loading control G was rat GAPDH.

Probe	FT+ (n=3)	FT- (n=3)	Ratio
Aα (2.3-kb)	2.12 ± 0.17	1.04 ± 0.07	2.0
Bβ (1.7-kb)	4.23 ± 0.51	1.23 ± 0.09	3.4
γ (1.5-kb)	0.63 ± 0.03	0.24 ± 0.05	2.6

Table 1. Densitometry values and analysis of quantitative Northern blot (figure 4.)

Characterization of fibrinogen expressed in FT+ mice

We examined plasma samples from FT+ and FT- animals by immunoblot analysis, using polyclonal antisera to the human protein. As depicted in Figure 5, fibrinogen in plasma from FT+ mice was indistinguishable from fibrinogen in plasma from FT- mice. With the anti-fibrinogen antibody, three bands were seen. The largest at approximately 59 kDa was as anticipated from the cDNA sequence of mouse Aα-chain, which encodes 538 amino acids (J. Degen, personal communication). The next band at 55 kDa was likely the mouse Bβ-chain; the smallest band at 50 kDa was slightly larger than the 48 kDa anticipated from the mouse γ-chain cDNA, which contains the same number of codons as the human cDNA. The band assignments were confirmed by cross-reactivity with chain-specific antibodies. Two bands reacted with the Aα-chain antibody, the 59 kDa band identified as the complete mouse Aα-chain, and a second strong band at 55 kDa overlapping Bβ-chain. A similar Aα-chain doublet is usually seen in human fibrinogen, where the smaller band is likely due to Aα-chain degradation. We concluded that the 55 kDa band seen in mice is a mixture of normal Bβ-chain and degraded Aα-chain. Only the 50 kDa band reacted with the γ-chain antibody, confirming that this band, though slightly larger than anticipated, was mouse γ-chain. The fibrinogen chains in FT+ plasma were indistinguishable from the chains in the FT- plasma. This result confirmed that the transgene contained all the normal genes and was inserted intact into the host genome.

We measured the concentration of functional fibrinogen in mouse plasma with a modified Clauss' method that determines concentration from the thrombin clotting time. The fibrinogen concentration in a plasma sample pooled from 11 C57Bl/6J mice was 1.3 mg/ml. Fibrinogen concentrations for three FT+ mice were 2.2, 2.0 and 1.9 mg/ml. As the values determined by the Clauss assay from the 3 FT+ mice were comparable to the average values determined by ELISA for line 1, we concluded that

the fibrinogen antigen in the FT+ mice is the same as the functional protein. Thus, the fibrinogen expressed from the transgenic locus has clotting activity similar to fibrinogen expressed for the normal locus.

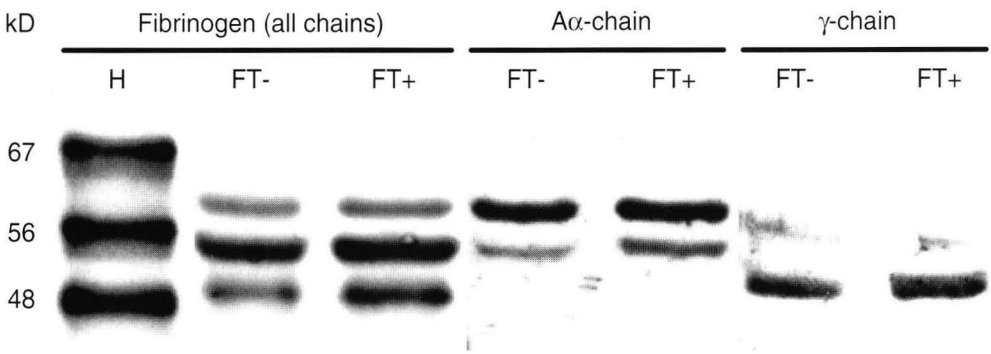

Figure 5. Western blot analysis of fibrinogen in mouse plasma.
Plasma samples from FT- and FT+ mice were run on an SDS-PAGE and detected with a polyclonal anti-human fibrinogen antibody, a polyclonal anti-human Aα-chain antibody, or a polyclonal anti-human γ-chain antibody. H, purified human fibrinogen, was run for comparison.

Plasma fibrinogen concentration as a function of age

Fibrinogen concentrations in 17 FT+ and 18 FT- animals were measured by ELISA once a month for 1 year. The results, shown in Figure 6, demonstrated that fibrinogen concentrations in the FT+ mice were significantly higher than in FT- mice ($p < 0.0001$) at all ages. Moreover, plasma fibrinogen concentrations in all mice, regardless of genotype, rose slightly but significantly ($p < 0.0001$; paired t-test) from month 2 to month 12 (FT+ 2.0 to 2.9 mg/ml; FT- 1.2 to 2.1 mg/ml).

Pathology

The FT+ positive mice demonstrated no increased mortality or morbidity as compared to normal littermates. While there was an approximately 12.5 % pre-sacrifice mortality rate amongst the 12-month-old animals, there was no correlation with genotype. Idiopathic dermatitis, which is common in the C57Bl/6J background (27), appeared in ~ 5 % of all line 1 animals, with no increased severity in the FT+

mice. In addition, there were no other lesions or extremity necrosis that might be consistent with a microthrombotic disorder. Animals were active and alert, demonstrating no behavioral differences. The five FT+ mice from the founder's first litter lived to 2+ years old, without any overt pathologic phenotype. At sacrifice, animals were perfusion fixed with 4 % paraformaldehyde. Necropsy examination of 17 FT+ and 18 FT- mice established that there were no gross abnormalities of the organs. We examined representative HE-stained sections of lung, liver, spleen and kidney from 3 FT+ and 3 FT- 12-month-old female mice. There were no significant histological differences in these organs.

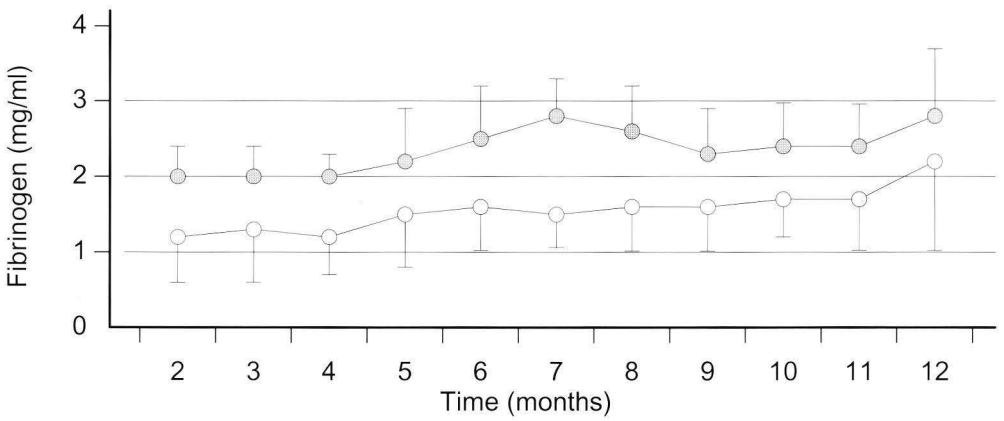

Figure 6. Changes in plasma fibrinogen concentrations in mice with time.
plasma was tested once a month by ELISA for 11 months (ages 2 through 12). Open circles are FT- (n=18); closed circles are FT+ (n=17).

Discussion

As a first step to examining the role of hyperfibrinogenemia in disease, we generated a mouse line with heritable hyperfibrinogenemia. In order to mimic the natural genetic control for fibrinogen synthesis, we used the complete fibrinogen locus as the transgene. Because we wished to study hyperfibrinogenemia in C57Bl/6J animals, we injected the genomic transgene into zygotes from this strain. We found that 2 out of 3 transgene positive animals had elevated levels of plasma fibrinogen. These experiments demonstrate that insertion of a large genomic transgene can promote

expression of multiple genes, in a manner consistent with expression from the endogenous locus. This is the first time that a multi-chain, disulfide-linked protein has been expressed for a single transgene.

In another experiment, we (unpublished results) isolated the genomic fragment from the P1 vector by restriction enzyme digestion, and injected this fragment into zygotes from C57Bl/6J X C3H/HeJ mice. Here we found that 2 out of 4 transgene-positive mice had elevated levels of plasma fibrinogen. Although our experiments were not designed to test the influence of the vector, comparing the results from the different transgenes suggests that removal of the P1 sequence was not critical for expression. This conclusion differs from published experiments, where removal of the vector sequences resulted in more reproducible patterns and much higher levels of expression of the β-globin transgene **(28, 29).** Perhaps the fibrinogen transgene contains not only the three genes of the fibrinogen locus, but also other control elements that mitigate the impact of the vector.

Quantitative Northern analysis of liver RNA showed that in the FT+ mice steady-state message levels were increased for all three genes, varying from 2-fold for the γ mRNA to 3.5-fold for the Bβ mRNA relative to levels in FT- mice. This increase in steady-state message from all three genes was similar to the 2-fold increase in plasma fibrinogen, suggesting that the steps of chain assembly and subsequent fibrinogen secretion were not limiting in the FT+ mice. The correlation between increased mRNA and increased fibrinogen was not found in transgenic mice that overexpress only the Bβ-chain gene. Because synthesis of the Bβ-chain was rate-limiting for fibrinogen synthesis in cultured human hepatoma cells, we generated mice using the complete fibrinogen Bβ-chain gene isolated from a mouse 129 Sv/EV genomic cosmid library **(30).** Northern blot analysis of liver RNA showed a 2- to 3-fold increase in Bβ mRNA in the transgenic mice relative to normal mice. Nevertheless, plasma fibrinogen levels in the transgenic mice did not differ from normal. We conclude that increased expression of all three genes is required to increase plasma fibrinogen levels.

Western blot analysis showed that plasma fibrinogen in FT+ mice was the same as plasma fibrinogen in FT- mice. Additionally, because fibrinogen concentrations measured by the ELISA assay and the modified Clauss assay were similar, fibrinogen expressed from the transgene appears functionally equivalent to normal. This suggest that all aspects of fibrinogen synthesis, like chain assembly and post-translational

modifications, from the transgene were normal. Thus, the FT+ animals of line 1 have normal, functional plasma fibrinogen at a concentration ~ 2-fold higher than normal mice of the same genetic background.

In humans there is no numerical definition of hyperfibrinogenemia, because different fibrinogen assays run on identical samples can result in very different concentrations **(31-33).** Usually, the term hyperfibrinogenemia is applied to fibrinogen concentrations above the normal range for the assay employed. In population studies, fibrinogen concentrations are often divided into tertiles, where people with plasma fibrinogen concentrations in the upper tertile are hyperfibrinogenemic **(5).** Using the ratio of the upper boundary of the central tertile to the lower boundary can eliminate the numerical differences between assays This also maximizes the difference between normal and elevated fibrinogen. Applying this method to 4 different population studies **(5),** hyperfibrinogenemia is 118-141 % of normal plasma fibrinogen. In our model, FT+ mice (2.0 mg/ml) have approximately 160 % of normal fibrinogen levels for a C57Bl/6J mouse (1.2 mg/ml). On the basis of these percentages, the FT+ mice exhibit substantial hyperfibrinogenemia. Nevertheles, the fibrinogen levels in the transgenic mice are in the normal range for humans, where 2-3 mg/ml is commonly considered to be normal. Thus, if the fibrinogen concentration per se, rather than a relative increase in concentration, is the risk factor, then the transgenic mice would not have an increased risk. This reasoning is logical, but only with the caveat that the reported concentrations should not be considered absolute, because they were determined using a functional assay desighned to measure human fibrinogen.

This significantly elevated concentration of plasma fibrinogen did not impact on the breeding or long-term survival of these mice. We therefore anticipate that we will be able to breed these mice with mice that model human disease, such as the apoE-deficient model of atherosclerosis **(34, 35)** and the NOD (non-obese diabetic) mouse model of diabetes mellitus. Studies with these models may determine whether hyperfibrinogenemia has a role in the initiation or progression of symptomatic disease **(36).**

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Chapter V

Overexpression of fibrinogen in apoE3-Leiden transgenic mice does not influence the progression of cholesterol-induced atherosclerosis

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ABSTRACT

Although hyperfibrinogenemia appears to be linked with atherosclerosis as shown by many epidemiological studies, it is not established whether elevated fibrinogen has an etiological role in the pathogenesis or is just a reflection of the ongoing disease.
In this paper, we have studied the contribution of fibrinogen to the development of atherosclerosis by cross-breeding atherosclerosis-prone apoE3-Leiden mice, which have a human-like lipid profile, with mice overexpressing fibrinogen. Genetic compound offspring were used to evaluate the progression of atherosclerotic lesions after being fed an atherogenic diet for 7 weeks.
It was observed that the lesion area of plaques as well as lesion severity in the aortic valve were not significantly different in apoE3-Leiden: mice overexpressing fibrinogen as compared to control apoE3-Leiden mice. No thrombus or fibrin deposition was observed in atherosclerotic lesions of either group of mice by histological Matrius-Scarlet-Blue staining.
These results indicate that elevated plasma fibrinogen concentrations in apoE3-Leiden transgenic mice do not affect the progression of diet-induced atherosclerotic lesions.

INTRODUCTION

The development of human atherosclerosis is a slow process. The process starts at an early age with the accumulation of lipid-rich foam cells in the arterial intima, macrophages and smooth muscle (SMC) cells becoming gradually involved in this process. These foam cell lesions can in turn progress into fibrofatty lesions and then to fibrous plaques, involving SMC migration from the media to the affected area in the intima. In later stages, plaques may rupture or fissure, leading to the formation of a thrombus. This advanced plaque can subsequently result in further plaque growth or in vascular occlusion, leading to myocardial infarction **(1-4).**
Elevated plasma fibrinogen has been shown in numerous epidemiological studies to be positively associated with a greater occurrence of cardiovascular disease **(5-11)** and atherosclerosis **(12-14)**. The cumulative evidence suggests that raised fibrinogen together with raised serum cholesterol increases the risk of cardiovascular disease incidence as compared to the individual risk factors **(10),** whereas, the risk associated with hypercholesterolemia remains low when combined with low fibrinogen

concentrations **(10).** To date, a causative relationship between increased plasma fibrinogen and atherosclerosis is uncertain. This uncertainty is enhanced by the fact that fibrinogen is an acute phase protein with concentrations rising in response to inflammation **(15, 16)**. Since atherosclerosis itself has often an inflammatory component **(1-4, 17, 18)**, it is believed that this may lead to elevated plasma fibrinogen concentrations. Data from literature suggest a critical role for inflammation in both atherogenesis and acute thrombosis **(1, 19, 20).** Whether fibrinogen may be considered as a simple marker or a real cause of atherosclerosis remains to be explored. With a transgenic mouse model we explored these two possibilities.

MATERIALS AND METHODS

Mice

Transgenic mice overexpressing fibrinogen were previously generated by conventional transgenesis technology **(21, 22).** Transgenic mice overexpressing human apoE3-Leiden have been previously described **(23)** and were a generous gift from L. Havekes (Gaubius Laboratory). Identification of apoE3-Leiden transgenic mice was performed by an ELISA for human apoE, as previously described **(24).** Genotypic identification of mice overexpressing fibrinogen was performed by polymerase chain reaction (PCR) analysis of genomic DNA from the tail. The PCR conditions have been previously described **(21, 22)**. Crosses were performed between mice overexpressing fibrinogen and apoE3-Leiden transgenic mice to generate the desired compound genotypes (apoE3-Leiden mice overexpressing fibrinogen). apoE3-Leiden transgenic mice were used as the control group in this study. Mice of 8-12 weeks of age were used in this study. All mice were maintained in a temperature-controled room, illuminated from 7 am to 7 pm, and were given free access to food and water throughout the experiment. The protocol of this study was approved by the Leiden University Ethics Commission for animal research (UDEC).

Diet

Before the start of the study, animals were kept on a standard mouse chow diet, and thereafter all mice were maintained on an atherogenic diet containing high saturated fat (15 % w/w), cholesterol (1 % w/w), and 0.5 % (w/w) cholate (N diet) (Hope Farms, Woerden, The Netherlands) for 7 weeks.

Histological assessment of atherosclerosis

After 7 weeks of being fed an atherogenic diet, mice were anesthetized and subsequently sacrificed. The entire heart plus aorta up to the diaphragm were dissected and fixed (24 hours, room temperature) with 2 % paraformaldehyde. The hearts were then sectioned just below the atria. The tissues were then dehydrated and paraffin-embedded. The hearts were sectioned perpendicularly to the axis of the aorta, beginning with the heart and working in the direction of the aortic arch as described by Paigen *et al* **(25).** Once the aortic root was identified by the appearance of the aortic valve leaflets, serial 5 µm sections were taken at 30 µm intervals and mounted on 3-AminoPropyl-3-Ethoxysilane-coated slides. Sections were air-dried for 48 hours at 37 °C and were then stained with hematoxylin-phloxin-Saffron (HPS). Per mouse, 4-5 sections with intervals of 30 µm were used for quantification of atherosclerotic lesions, using a computer image analysis (Leica).

The lesions were classified into five categories (type I-V) as previously described **(24, 26)** : I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, V) severe plaque. Per mouse, four slides were used to calculate the lesion severity with the following formula (total number of each lesion type per mouse group / number of mice per genotype x 4).

Mouse macrophages were immunostained with AIA31240 (1:3000, Accurate Chemical and Scientific, New York, USA); smooth muscle cells were stained with α-actin (1A-4, Roche, Almere, The Netherlands). Matrius-Scarlet-Blue (MSB) **(27)** was used to stain the fibrin and connective tissues in mouse sections **(27)**. Sirius red (SR) as well as HPS were also used to stain connective tissues **(27).** Histological elastin staining (Weigert's elastica method) was used to delineate the border between media and intima **(27).**

Plasma fibrinogen analysis

Whole blood was collected into chilled tubes coated with EDTA (Sarstedt, Etten-Leur, The Netherlands) from the tail vein of mice either before the study, or after 7 weeks on the atherogenic diet. Mouse plasma fibrinogen concentrations were measured by sandwich ELISA, using purified polyclonal rabbit anti-rat fibrin antibodies **(28)** for coating and purified polyclonal goat anti-mouse fibrinogen antibodies conjugated to peroxidase as conjugate (Nordic, Tilburg, The Netherlands). The detection was performed by the immunoperoxidase procedure using tetramethylbenzidine (TMB) as a substrate (Organon Teknika, Boxtel, The Netherlands). The fibrinogen concentration of pooled citrated plasma from mice was determined by gravimetrical analysis **(29)**. This pooled plasma was subsequently used as the standard in ELISA assays.

Plasma cholesterol analysis

Total cholesterol levels were measured in the plasma of all mice enzymatically, using a commercially available kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Platelet aggregation analysis

Whole blood (generally 700-900 µl per mouse) from apoE3-Leiden mice overexpressing fibrinogen, apoE3-Leiden transgenic mice as well as mice overexpressing fibrinogen after being fed an atherogenic diet for 7 weeks, was drawn directly into citrate anticoagulant from the retro-orbital of anesthetized mice. Blood samples were immediately diluted 1:1 with phosphate-buffered-saline, and aggregation assays were performed at 37 °C using a chronolog 590 whole blood aggregometer (Chronolog cooperation, USA). Aggregation was initiated by the addition of 1 µl of 1 mg / ml equine collagen (Kordia, Leiden, The Netherlands) to 500 µl of platelet suspensions.

Statistical analysis

Results are reported as mean ± SEM. Statistical differences were determined by Student's t test.

RESULTS

Atherosclerotic lesion area and lesion severity

To investigate whether the variation in endogeneous plasma fibrinogen concentrations significantly change the process of atherosclerosis, we determined the effect of overexpression of fibrinogen in a well-established model for atherosclerosis, apoE3-Leiden transgenic mice. From mice overexpressing fibrinogen and apoE3-Leiden mice with elevated blood cholesterol we obtained double transgenic mice with a combined phenotype. We observed an approximately 40 % increase in plasma fibrinogen concentration on both basal chow diet and atherosclerotic diet and 4-8-fold increase in plasma cholesterol concentration on atherosclerotic diet as compared with the respective single transgenic mice.

Genotypes	(n)	Plasma fibrinogen (mg/ml) Chow diet	Atherogenic diet	Plasma cholesterol (mmol/l)	Lesion area (μm^2 x 1000)	Lesion Type I - II	III - V
APOE3-Leiden (F)	4	1.49 ± 0.02	3.30 ± 0.04	22.2 ± 0.7	49.4 ± 8.1	0.75	1.4
			P < 0.005				
APOE3-Leiden:Fbg ovex (F)	4	2.29 ± 0.04	4.40 ± 0.06	24.2 ± 2.2	47.8 ± 7.2	0. 5	1.5
Fbg ovex (F)	3	2.26 ± 0.07	4.50 ± 0.05	3.0 ± 0.6	0.0	0.0	0.0
APOE3-Leiden (M)	4	1.70 ± 0.03	3.27 ± 0.05	19.7 ± 2.7	22.5 ± 6.3	0.75	1.3
			P < 0.001				
ApoE3-Leiden:Fbg ovex (M)	3	2.27 ± 0.04	4.20 ± 0.06	20.7 ± 0.7	26.3 ± 5.8	0.75	1.7
Fbg ovex (M)	5	2.28 ± 0.02	3.90 ± 0.04	4.8 ± 0.7	0.0	0.0	0.0

		Platelet aggregation	
Genotypes	(n)	Amplitude (ohms)	Slope (ohms/min)
APOE3-Leiden (F,M)	5	19.7 ± 0.9	11.8 ± 1.1
APOE3-Leiden:Fbg ovex (F,M)	5	18.6 ± 0.7	13.6 ± 1.1
Fbg ovex (F,M)	8	16.4 ± 1.1	10.4 ± 1.1

Table 1. Characteristics of different mouse genotypes after the consumption of an atherogenic diet.
Lesion area, lesion severity in the aortic valve, fibrinogen concentrations as well as cholesterol in plasma, and platelet aggregation in the whole blood were determined either in double transgenic mice overexpressing fibrinogen and expressing the apoE3-Leiden transgene, mice only overexpressing fibrinogen or single apoE3-Leiden transgenic mice (control group). Plasma fibrinogen levels were determined in mice fed a chow diet and after 7 weeks of consumption of an atherogenic diet. For details see Materials and Methods. Values for all measurements are expressed as the mean ± SEM of n (n = number of mice per group). $P < 0.05$ was accepted as statistically significant (t-test). The abbreviations in table 1 are: F (female), M (male), Fbg (fibrinogen), and ovex (overexpression).

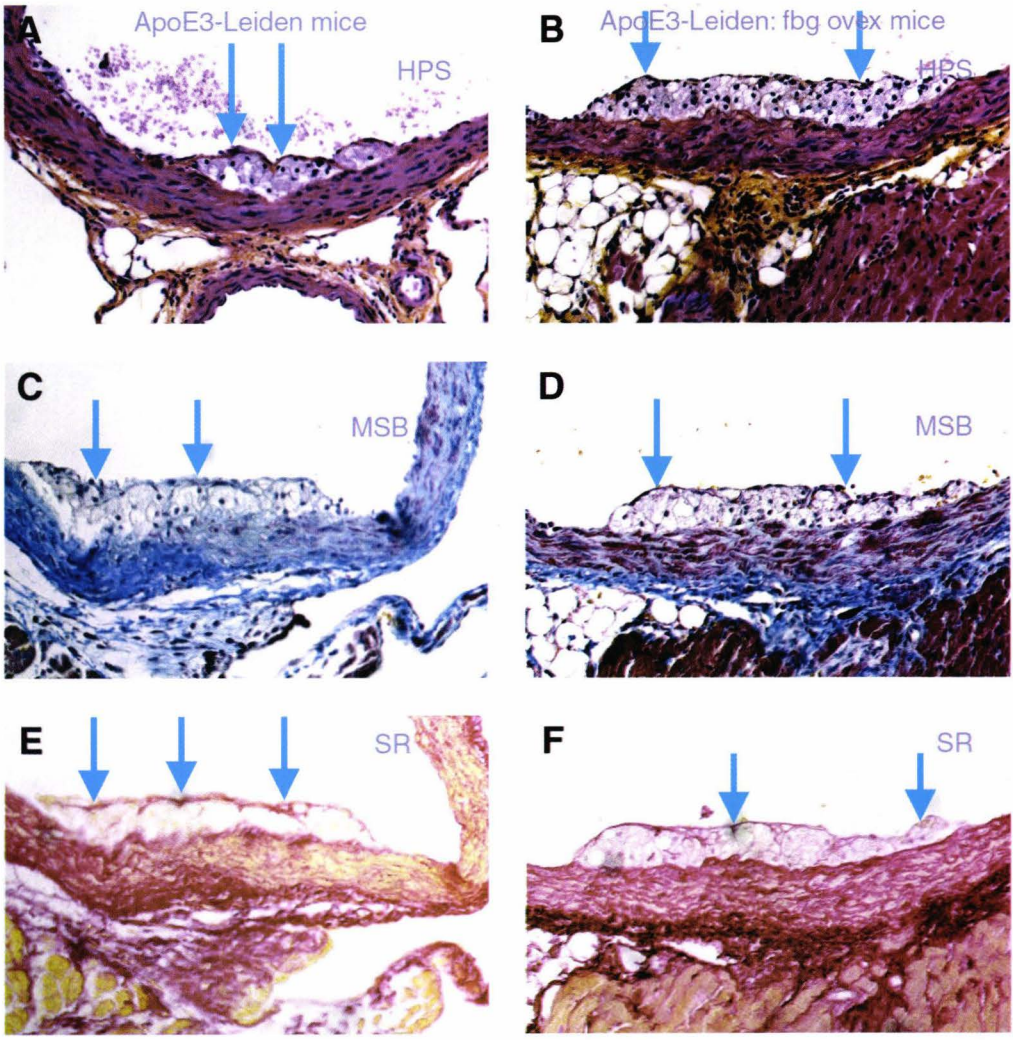
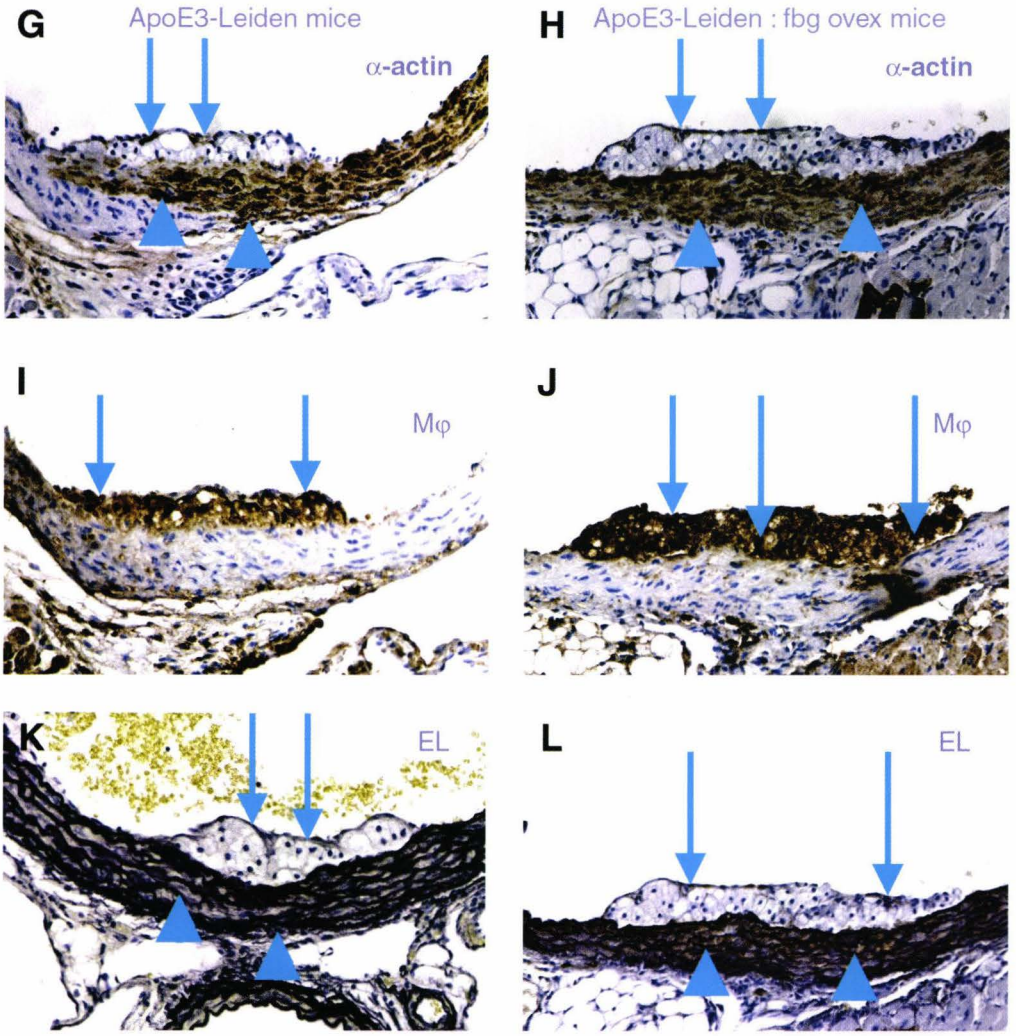


Figure 1. Representative photomicrographs (magnification 20x) of atherosclerotic lesions in the aortic valve of mice overexpressing fibrinogen combined with apoE3-Leiden transgene and apoE3-Leiden transgenic mice after the consumption of an atherogenic diet.

Consecutive slides of a mild atherosclerotic plaque (type III) of an apoE3-Leiden mouse (**A, C, E, G, I, K**) compared to a mild plaque (type III) of an apoE3-Leiden mouse overexpressing fibrinogen (**B, D, F, H, J, L**). Collagen was stained with Haemotoxylin Phloxin Saffron (HPS) (**A, B**), Martius Scarlet Blue (MSB) (**C, D**) and Sirius red (SR) (**E, F**). Collagen deposition within the fibrous cap of apoE3-Leiden mice (**A, C**, and **E**) as well as combined mice (apoE3-Leiden: mouse overexpressing fibrinogen) (**B, D**, and **F**) was indicated by large arrows (intensely yellow-, blue-, and red-staining material respectively). No fibrin deposition was observed in either mouse group using MSB staining (**C, D**). Mouse smooth muscle cells were immunostained with α-actin (1A4) (**G, H**). Arrowheads indicate accumulation of smooth muscle cells in media and some in the fibrous cap (large arrows, brown staining) of control group (apoE3-Leiden transgene) (**G**) and of apoE3-Leiden mice overexpressing fibrinogen (**H**). Mouse macrophages were immunostained with AIA31240 (Mφ) (**I, J**, brown staining). The plaque of apoE3-Leiden : mice overexpressing fibrinogen as well as the control group was covered with



foam cells (**I, J**, large arrows). Elastic fibers were stained with Weigert's Resorcin Fuchsin (EL) (**K, L**). Arrowheads in **K** and **L** (intensely violet to black staining material) indicated intact elastic fiber in the media of combined mice as well as the control group. Some elastic fibers occur in the fibrous cap of either mouse groups (Large arrows, violet to black staining).

Despite these clear changes in plasma concentration no significant changes in atherosclerotic lesion area nor severity of the lesions were observed (table 1). A more pathological analysis of the lesions in double transgenic mice as compared with the single transgenic apoE3-Leiden founders was performed. No differences with respect to collagen deposition in the plaques observed between the groups (figure 1 A-F). In

none of the mice were fibrin deposition or thrombus observed (figure 1 C, D). Similarly no differences in smooth muscle cell proliferation and migration (figure 1 G, H), macrophage presence (figure 1 I, J), and elastin damage (figure 1 K, L) were seen.

Platelet aggregation in mice overexpressing fibrinogen

To determine whether the observed increase in plasma fibrinogen concentrations alters the rate and / or amplitude of platelet aggregation, platelet aggregation was measured in different mouse genotype groups fed the N diet. Despite significant differences in absolute concentrations of plasma fibrinogen between different mouse genotype groups (apoE3-Leiden mice overexpressing fibrinogen, and the control group apoE3-Leiden transgenic mice), the slope and amplitude of platelet aggregation did not differ significantly from each other (table 1).

Discussion

Although an elevated plasma fibrinogen level is associated with the severity of atherosclerosis in many epidemiological studies, it has not yet been established which comes first, high plasma fibrinogen levels or atherosclerosis. Because it is not possible to answer this question in human epidemiological studies, we developed a transgenic mouse model to examine these two possibilities.

We bred double transgenic mice that have a combined phenotype, and these have an increased plasma fibrinogen level and an increased serum cholesterol level, as expected (table 1). The plasma fibrinogen level further increases upon consuming an atherogenic diet as observed previously **(30).**

Despite the clearly increased plasma fibrinogen levels no differences were seen in either lesion area or severity of atherosclerotic plaques between double transgenic mice and single transgenic apoE3-Leiden mice. These results suggest that in mice, fibrinogen does not causally contribute to the development and progression of atherosclerosis. Also studies in fibrinogen (knock-out) and apoE-deficient mice **(31)** and studies in fibrinogen-overexpression mice **(21, 22)** have not observed any effect of the plasma fibrinogen concentration on diet-induced atherosclerosis.

Previously, the characteristics of atherosclerosis have been widely studied in the apoE3-Leiden mouse model, and this model is generally considered to be a good model for human atherosclerosis, especially because the plaque composition is very similar to that of human plaques. In this study we observed that there was no fibrin deposition, neither in the double transgenic mice nor in the single transgenic apoE3-Leiden mice. Several mechanisms that may explain the causal contribution of fibrinogen and fibrin to atherosclerosis assume that there is fibrin deposition in the plaques. In human atherosclerotic plaques frequently fibrin deposition is observed **(32, 33)** suggesting that thrombosis is a critical mechanism in the pathogenesis of human atherosclerosis in contrast with atherosclerosis in the mouse model used. The difference in human atherosclerosis and atherosclerosis in the currently available mouse models might be an explanation of the total absence of stroke or myocardial infarction, so characteristic of the disease in man **(34, 35),** in the mouse models. The fibrin depositions in the plaques could contribute to chemotaxis of smooth muscle cells and monocytes/macrophages **(36, 37),** or provide a provisional matrix. These mechanisms cannot be studied in mouse models, in which no fibrin depositions appear to occur.

Other, possible causal mechanisms that are independent of fibrin deposition and thus can be studied in mouse models are the effect of the plasma fibrinogen concentration on the plasma viscosity, and platelet functions. The results of our study indicate that these mechanisms are unlikely to make an important contribution to atherosclerosis progression in mice.

In conclusion, a high plasma concentration of fibrinogen is unlikely to make a major contribution to the diet-induced atherosclerosis in mice, and the relation between high plasma fibrinogen and human cardiovascular disease remains unclear.

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Chapter VI

Genetic deletion of tissue-type plasminogen activator (t-PA) in APOE3-Leiden mice reduces progression of cholesterol-induced atherosclerosis

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ABSTRACT

There is no established evidence for the involvement of the plasminogen / plasmin system in the development of atherosclerosis, although epidemiological, genetic and molecular evidence suggest that plasminogen / plasmin may be involved in vascular disease.

In this paper, we have studied the contribution of the plasminogen / plasmin system in the development of atherosclerosis by cross-breeding apoE3-Leiden mice, which have a human-like lipid profile, with mice deficient in PAI-1, u-PA, and t-PA. Genetic compound offspring was used to evaluate the progression of atherosclerotic lesions after they were fed a mild atherogenic diet for 12 weeks.

Lesion area of plaques in the aortic valve was not significantly different in apoE3-Leiden : PAI-1 -/- and apoE3-Leiden : u-PA -/- mice as compared to apoE3-Leiden mice. In contrast, a significant 70 % reduction of the lesion area was observed in apoE3-Leiden : t-PA -/- mice as compared to control group apoE3-Leiden mice. In addition the early, regular fatty streaks and mild plaques increased in apoE3-Leiden : t-PA -/- mice, whereas the severe plaques (type IV and V) decreased in these animals. A lower deposition of collagen was observed in the atherosclerotic lesions of apoE3-Leiden : t-PA -/- mice as compared with apoE3-Leiden mice. Our results indicate for the first time that t-PA deficiency delayed the atherosclerotic process in this mouse model. The mechanisms whereby t-PA deficiency delays the atherosclerotic process are suggested to be through the loss of plasmin-mediated extracellular matrix degradation, inhibition of plasmin-mediated TGF-β, and zymogen matrix metalloproteinase (MMP) activation in the vessel wall (particularly decrease of collagen synthesis), and loss of mitogenic activity of t-PA on SMCs.

Key words: Fibrinolysis, SMC, Collagen, Atherosclerosis, Transgenic mice

INTRODUCTION

The development of human atherosclerosis is a slow process. The process starts at an early age with the accumulation of lipid-rich foam cells in the arterial intima, macrophages and smooth muscle (SMC) cells becoming gradually involved in this process. These foam cell lesions can in turn progress into fibrofatty lesions and then to fibrous plaques, involving SMC migration from the media to the affected area in

the intima. In later stages, plaques may rupture or fissure, leading to the formation of a thrombus. This advanced plaque can subsequently result in further plaque growth or in vascular occlusion, leading to myocardial infarction **(1-4).**

The plasminogen / plasmin activation cascade is involved in a wide variety of pathological and physiological processes, such as the generation of biologically active fibrin degradation products (FDPs), the activation of zymogens of the matrix metalloproteinase family, the degradation of extracellular matrix components, the activation of latent growth factors such as transform growth factor-β (TGF-β), cell migration and proliferation, tumor cell invasion and metastatic spread **(5-10).** Thus, plasmin-mediated proteolysis may be involved in the atherosclerotic process through several distinct mechanisms. The interplay between plasmin and TGF-β is particularly intriguing, because this growth factor is a known autocrine inhibitor of smooth muscle cell (SMC) proliferation and migration, as well as an inducer of extracellular matrix accumulation including collagen synthesis **(11-15).**

Increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) are considered a risk factor for myocardial infarction and recurrent infarction **(16-19),** and are associated with the progress of coronary artery disease **(20-22).** However, this latter association has not been supported by other studies **(23-25).**

A high level of tissue-type plasminogen activator (t-PA) antigen has also been shown to be associated with the presence of coronary heart disease **(19, 23, 26-29),** and a greater risk of future myocardial infarction **(23, 26),** and stroke **(27, 30, 31).** An increased level of t-PA and urokinase-type plasminogen activator (u-PA) in plaques suggests a possible role for increased plasmin proteolysis in atherosclerosis **(5-7, 9, 10, 32-34).** Plasmin-mediated proteolysis could be involved in neovascularization of plaques, the stimulation of plaque rupture, ulceration and aneurysm formation **(32-34).** Up to now a causal relationship between the plasminogen system and these processes has not been established except for aneurysm formation **(35).**

To explore the hypothesis that variations in endogenous fibrinolytic activity significantly alter the process of atherosclerosis, we examined the effect of genetic modification of PAI-1, u-PA, and t-PA expression in a well-established model of atherosclerosis, apoE3-Leiden mice. We used the apoE3-Leiden mice as atherosclerosis-prone mice, because the lipoprotein profiles of these mice are very similar to the ones found in humans and they develop human-like atherosclerotic lesions in time **(36).** These atherosclerosis-prone mice were cross-bred with PAI-1 -/-, u-PA -/-, and t-PA -/- mice, and genetic compound offspring evaluated for

atherosclerosis progression after consumption of a mild atherogenic diet for 12 weeks. Furthermore, these mice are highly susceptible to high fat diet-induced atherosclerosis **(36).** We used a mild atherogenic diet, because this does not lead to abnormalities in the liver **(37).**

Materials and Methods

Mice

Mice deficient in t-PA, u-PA, and PAI-1 previously generated by homologous recombination were a generous gift from Drs. D. Collen and P. Carmeliet (University of Leuven, Leuven, Belgium) **(38, 39).** Transgenic mice overexpressing human apoE3-Leiden have been previously described **(40)** and were a generous gift from Dr. L. Havekes (Leiden University and the Gaubius Laboratory (TNO-PG), Leiden, The Netherlands). Identification of apoE3-Leiden transgenic mice was performed by an ELISA for human apoE, as previously described **(36).** Genotypic identification of t-PA, u-PA, and PAI-1 mice was performed by polymerase chain reaction analysis of genomic DNA from the tail. The polymerase chain reaction conditions have been previously described **(38, 39)**. Crosses were performed between t-PA, u-PA, or PAI-1 (-/-) and apoE3-Leiden mice to generate the desired compound genotypes (apoE3-Leiden : t-PA -/-, or +/-), (apoE3-Leiden : u-PA -/-, or +/-), (apoE3-Leiden : PAI -/- or +/-). ApoE3-Leiden transgenic mice were used as the control group in this study and were obtained from crosses between two groups of heterozygous mice of each genotype. Female mice of different genotypes of 8-12 weeks of age were used in this study. All mice were maintained in a temperature-controled room, illuminated from 7 am to 7 pm, and were given free access to food and water throughout the experiment. The protocol of this study was approved by the Leiden University Ethics Commission for animal research (UDEC).

Diet

Before the start of the study, animals were kept on a standard mouse chow diet, and thereafter all mice were maintained on an atherogenic diet containing high saturated fat (15 % w/w), cholesterol (1 % w/w), and cholate (0.1 % w/w) (P diet) (Hope Farms, Woerden, The Netherlands) for 12 weeks. No macroscopic abnormalities of the livers were observed after consumption of the P diet.

Histological assessment of atherosclerosis

After 12 weeks of being fed the atherogenic P diet, mice were anesthetized and subsequently sacrificed. The entire heart plus aorta up to the diaphragm were dissected and fixed (24 hours, room temperature) with 2 % (w/v) paraformaldehyde. The hearts were then sectioned just below the atria. The tissues were dehydrated and paraffin-embedded. The hearts were sectioned perpendicularly to the axis of the aorta, beginning with the heart and working in the direction of the aortic arch as described by Paigen *et al* **(41).** Once the aortic root was identified by the appearance of the aortic valve leaflets, serial 5 µm sections were taken at 30 µm intervals and mounted on 3-AminoPropyl-3-Ethoxysilane (APES)-coated slides. Sections were air-dried for 48 hours at 37 °C and were subsequently stained with hematoxylin-phloxin-Saffron (HPS). Per mouse, 4-7 sections with intervals of 30 µm were used for quantification of atherosclerotic lesions, using a computer image analysis (Leica).

For determination of the severity of atherosclerosis, the lesions were classified into five categories (type I-V) as previously described **(36, 42)** : I) early fatty streak, II) regular fatty streak, III) mild plaque IV) moderate plaque, V) severe plaque. Per mouse, four slides were used to calculate the lesion severity. Thus, the different lesion types of each mouse group were calculated as follows: (total number of each lesion type per mouse group) / (number of mice per genotype x 4).

Mouse macrophages were immunostained with AIA31240 (1:3000, Accurate Chemical and Scientific, New York, USA); smooth muscle cells were stained with α-actin (1A-4, Roche, Almere, The Netherlands). Matrius-Scarlet-Blue (MSB) **(43)** was used to stain the fibrin and connective tissues in mouse sections **(43)**. Sirius red (SR) as well as HPS were also used to stain connective tissues **(43).** Histological elastin staining (Weigert's elastica method) was used to delineate the border between media and intima **(43).**

Plasma cholesterol analysis

Total cholesterol levels were measured in the plasma of all mice enzymatically, using a commercially available kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Statistical analysis

Results are reported as mean ± SEM. Statistical differences were determined by Student's test (t-test)

Results

Atherosclerotic lesion area and lesion severity

To investigate whether the variations in endogeneous fibrinolytic parameters significantly change the process of atherosclerosis, we determined the effect of genetic modification of t-PA, u-PA, and PAI-1 in a well-established model of atherosclerosis, apoE3-Leiden transgenic mice. As depicted in table 1 and figure 1, no significant changes were observed with respect to the atherosclerotic lesion area between different combined genotypes of either u-PA or PAI-1 deficiency with apoE3-Leiden mice, and control animals (apoE3-Leiden). In contrast, we observed a significant ($p < 0.003$) approximately 70 % reduction of lesion area in the apoE3-Leiden : t-PA (-/-) combined genotype, but not in the apoE3-Leiden : t-PA (+/-) combined genotype, as compared to the control group (apoE3-Leiden) (figure 1 and table 1). Furthermore, this reduction of lesion area in the t-PA (-/-) combined with apoE3-Leiden mice is independent of the concentration of cholesterol in the plasma (table 1).

We also evaluated the effect of the different genotypes on the severity of the atherosclerotic lesions in mice. The atherosclerotic lesion scoring has been ranked by one investigator (pathologist, **36, 42**) who was unaware of the genotype of the animals. As shown in table 1 and figure 2, most severe plaques (type IV and V) were observed in the control mice (apoE3-Leiden mice). In contrast, apoe3-Leiden : t-PA -/- mice demonstrated mostly fatty streaks to mild plaques (type I-III) (table 1 and figure 2). The differences between the compound genotype of apoE3-Leiden : t-PA -/- and control group (apoE3-Leiden) with respect to severe plaques (type IV and V)

and fatty streak lesions (type I and II) were found to be statistically significant (figure 2). Although type IV and V form the minority in apoE3-Leiden : t-PA -/-, the majority of these plaques occurred in just one mouse of this group.

Genotypes	(n)	PCHOL (mmol/l)	Lesion area (μm^2 X 1000)		Lesion Type I + II	III	IV + V
APOE3-Leiden	5	36.3 ± 3.4	191.1 ± 30.3		0.1	0.7	1.8
			P < 0.6				
APOE3-Leiden: PAI-1 + / –	10	35.3 ± 2.5	172.6 ± 15.6	P < 0.4	0.1	0.5	2.2
APOE3-Leiden: PAI-1 – / –	8	35.8 ± 1.8	157.7 ± 16.7		0.7	0.7	1.8
APOE3-Leiden	9	34.5 ± 1.8	176.8 ± 26.8		0.1	0.6	1.8
			P < 0.4				
APOE3-Leiden: u-PA + / –	9	32.6 ± 2.5	151.4 ± 13.4	P < 0.4	0.3	0.6	1.6
APOE3-Leiden: u-PA – / –	10	35.9 ± 3.0	149.8 ± 15.9		0.3	0.5	1.6
APOE3-Leiden	7	30.5 ± 2.2	164.1 ± 26.3		0.1	0.6	1.9
			P < 0.5				
APOE3-Leiden: t-PA + / –	11	31.1 ± 1.7	141.6 ± 12.1		0.1	0.5	1.7
				P < 0.003			
APOE3-Leiden: t-PA – / –	9	29.2 ± 1.0	46.9 ± 8.1		0.8	0.9	0.6

Table 1. Characteristics of different mouse genotypes after the consumption of a mildly atherogenic diet for 12 weeks.
Lesion area and lesion severity in the aortic valve as well as cholesterol in plasma were determined either in mice deficient in PAI-1, u-PA, or t-PA combined with the apoE3-Leiden transgene or apoE3-Leiden transgenic mice (control group). Per mouse, 4-7 sections were used for the quantification of atherosclerotic lesions. Per mouse, 4 sections were used to determine the severity of atherosclerosis. Plasma cholesterol levels (PCHOL) and lesion area are expressed as mmol/l and μm^2 x 1000 respectively. Lesion severity was expressed as (the total number of each lesion type per mouse group) / (number of mice per group x 4). Values for all measurements are expressed as the mean ± SEM of n (n = number of mice per group). $P < 0.05$ was accepted as statistically significant (t-test).

Pathological analysis

In figure 3, A-L shows representative pictures of atherosclerotic lesions in the different genotypes of t-PA (wild type t-PA (+/+) and homozygous (-/-)) combined with the apoE3-Leiden transgen, illustrating the differing severity of atherosclerotic plaques.

No fibrin was observed in the atherosclerotic plaques of any of the mice studied after histological MSB staining of aorta sections (figure 3 C, D, data shown for apoE3-Leiden and t-PA -/-).

The plaque formation was severe in the apoE3-Leiden mice, so consequently more collagen was observed in these plaques as seen in the HPS, MSB and SR (figure 3 A, C, E). In the severe plaques, the SMC could be replaced by fibroblasts (figure 3 G, H). The small plaques of the apoE3-Leiden : t-PA-/- mice contain mostly macrophages (figure 3 J). However, many macrophages were also observed (figure 3 I) in the

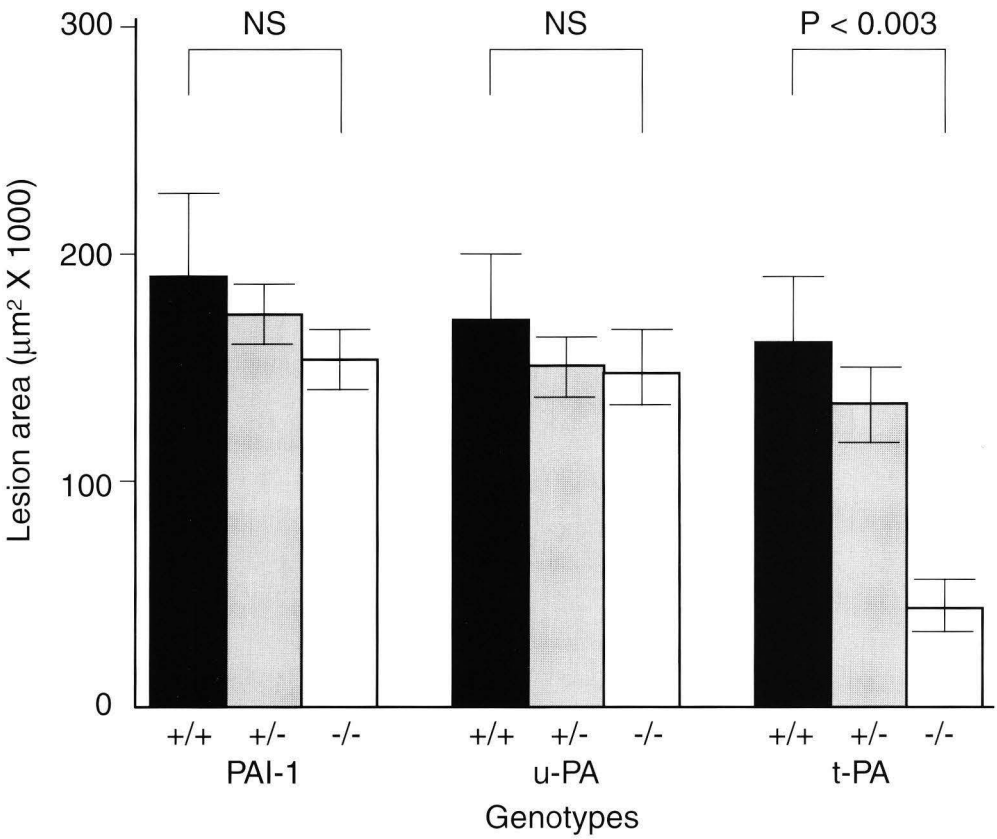


Figure 1. Effect of PAI-1, u-PA and t-PA gene deletion on atherosclerotic lesion area after the consumption of a mild atherogenic diet for 12 weeks.

Lesion area in the aortic valve was determined either in mice deficient in PAI-1, u-PA, t-PA combined with apoE3-Leiden mice or apoE3-Leiden mice (control group). Solid bars indicate apoE3-Leiden mice (control group). Per mouse, 4-7 sections were used for the quantification of atherosclerotic lesions. Values for all measurements are expressed as the mean ± SEM of n (n = number of mice per group, see table 1). $P < 0.05$ was accepted as statistically significant (t-test).

severe plaques of the apoE3-Leiden mice. As depicted in figure 3 K, the elastin in the severe plaques (type V lesion) of the apoE3-Leiden mice was damaged, indicating a thinning of the media and aneurysm formation in the atherosclerotic lesion area.

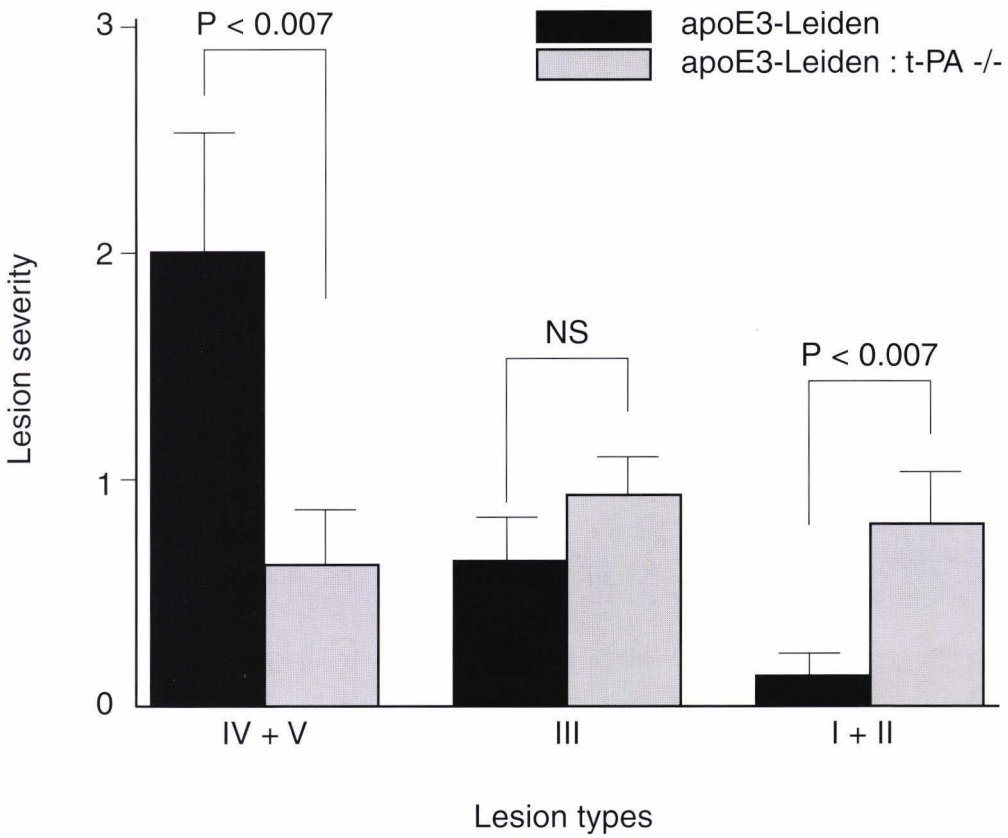


Figure 2. Effect of t-PA gene modification on the severity of atherosclerotic lesion after the consumption of an atherogenic diet for 12 weeks.
ApoE3-Leiden mice are indicated by solid bars and apoE3-Leiden : t-PA -/- mice by crosshatched bars. Type 1 lesion = early fatty streak, type 2 = regular fatty streak, type III = mild plaque, type IV = moderate plaque, type V = severe plaque. Type I lesion and type II are combined with each other as well as type IV and type V. Per mouse, 4 sections were used to determine of severity of atherosclerosis. Lesion severity was expressed as (the total number of each lesion type per mouse group) / (number of mice per group x 4). Values for all measurements are expressed as the mean ± SEM of n (n = number of mice per group, see table 1). $P < 0.05$ was accepted as statistically significant (t-test).

Although no significant differences were observed between the compound genotypes of PAI-1 (+/- or -/- combined with apoE3-Leiden) and control group, remarkably, necrosis of the macrophages associated with granulocyte infiltration was observed in plaques of 4 out of 8 apoE3-leiden : PAI-1 -/- mice compared to 1 out of 5 apoE3-Leiden mice, as shown by HPS staining (figure 4).

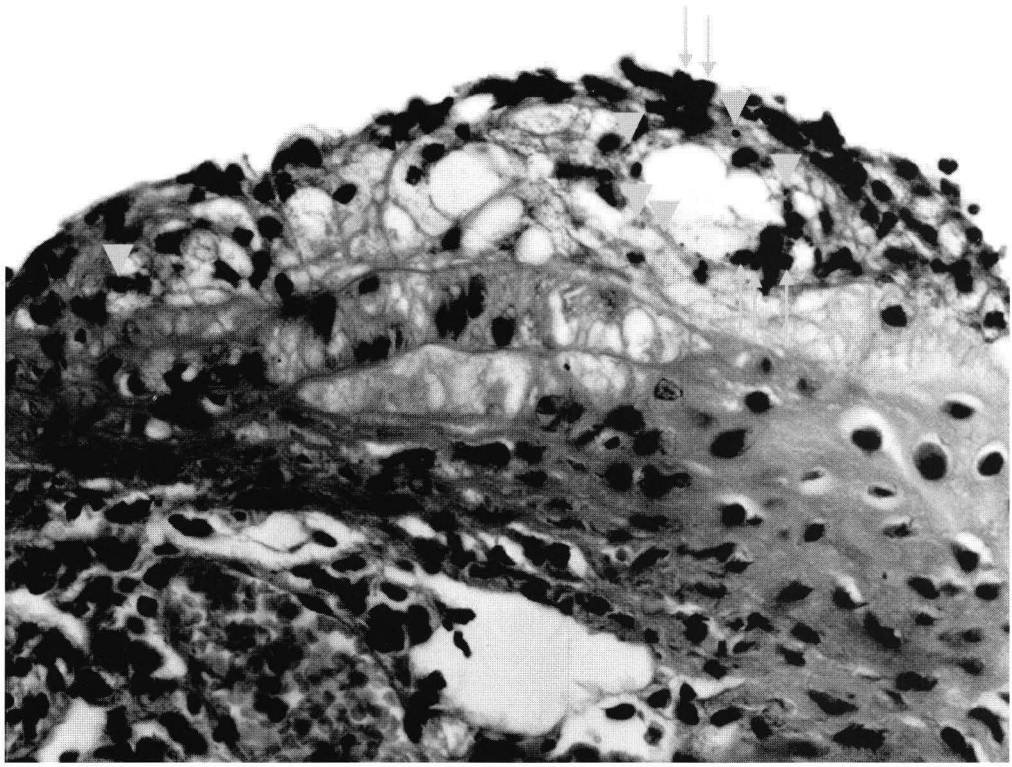


Figure 4. Representative photomicrograph (magnification 40x) of atherosclerotic lesions in the aortic valve of mice deficient in PAI-1 (-/-) combined with apoE3-Leiden transgenic after the consumption of a mildly atherogenic diet.
The necrosis in the plaque of the apoE3-Leiden : PAI-1 -/- mouse is indicated by thick arrowheads, and granulocytes by large arrows, using Haemotoxylin Phloxin Saffron (HPS) staining.

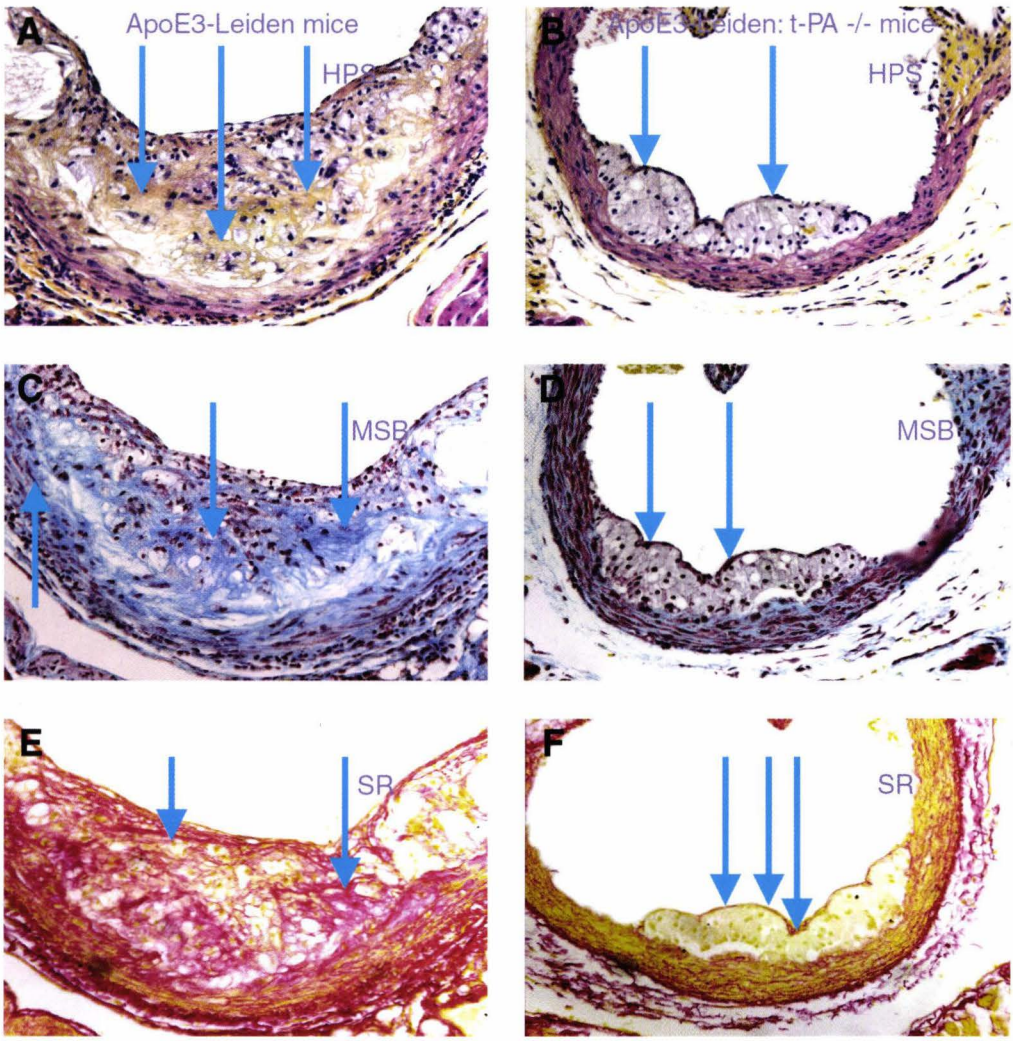
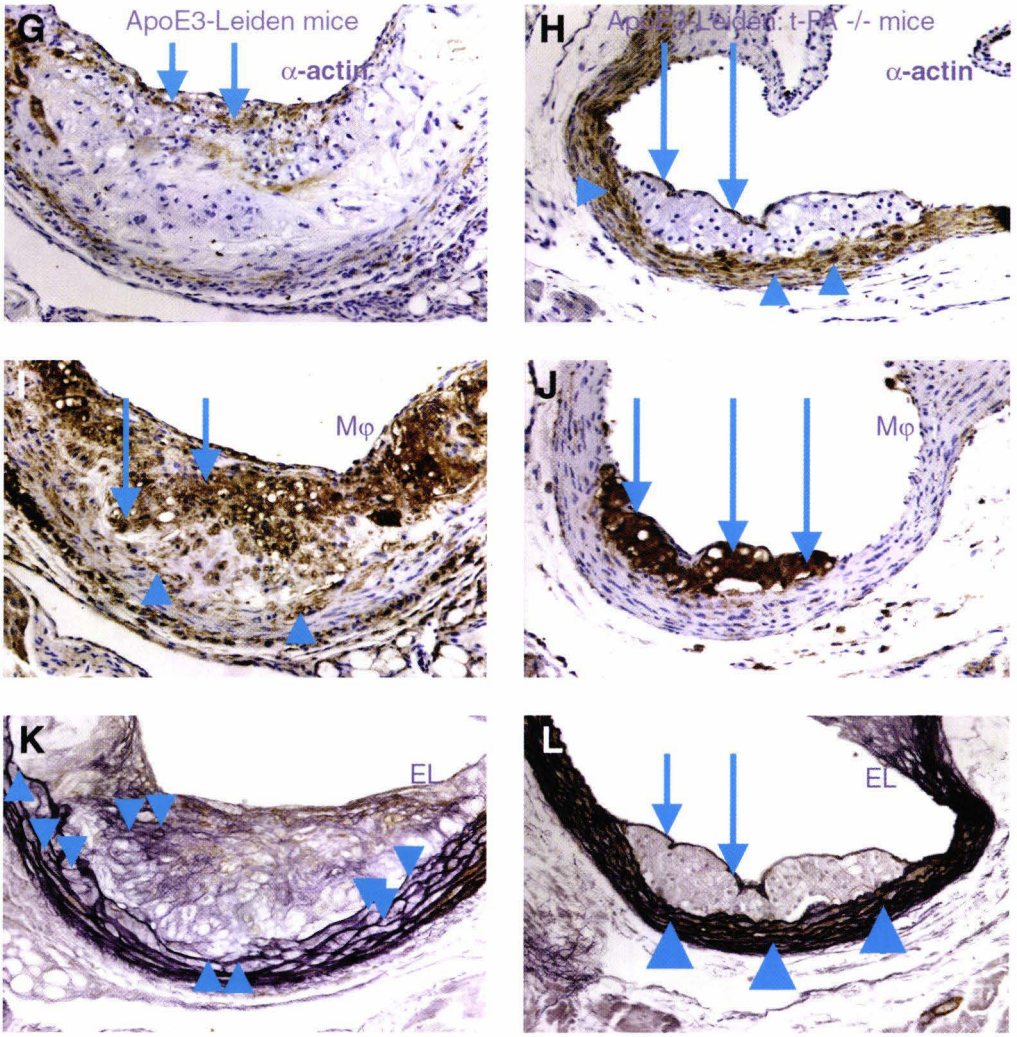


Figure 3. Representative photomicrographs (magnification 20x) of atherosclerotic lesions in the aortic valve area of mice deficient in t-PA combined with apoE3-Leiden transgene and apoE3-Leiden transgenic mice after the consumption of an atherogenic diet.

Consecutive slides of a severe atherosclerotic plaque (type V) of an apoE3-Leiden mouse (**A, C, E, G, I, K**) compared to a mild plaque (type III) of a apoE3-Leiden : t-PA -/- mouse (**B, D, F, H, J, L**). Collagen was stained with Haemotoxylin Phloxin Saffron (HPS) (**A, B**), Martius Scarlet Blue (MSB) (**C, D**) and Sirius red (SR) (**E, F**). Collagen deposition within the intima of apoE3-Leiden mice is indicated by large arrows in **A, C**, and **E** (intensely yellow-, blue-, and red-staining material respectively). The fibrous cap of apoE3-Leiden : t-PA -/- mice is highlighted by large arrows in **B, D, F, H,** and **L**. No fibrin was observed in either mouse group using MSB staining (**C, D**). Mouse smooth muscle cells were immunostained with α-actin (1A4) (**G, H**). Arrowheads indicate accumulation of smooth muscle cells in media and some in the fibrous cap (large arrows, brown staining) of t-PA -/- : apoE3-Leiden mice (**H**). The majority of smooth muscle cells were transformed into fibroblasts and some remained within the intima of apoE3-Leiden mice (**G**, large arrows. Mouse macrophages were immunostained with AIA31240 (Mφ) (**I, J**, brown staining). The intima of apoE3-Leiden : t-PA -/- mice was covered with foam cells (**I, J**, large arrows). Arrowheads and large arrows in **I** indicate the migration of macrophages from media into



the intima and the accumulation of macophages within the intima of apoE3-Leiden mice respectively. Elastic fibers were stained with Weigert's Resorcin Fuchsin (EL) (**K, L**). Large arrows in K (intensely violet to black staining material) indicated elastic fiber deposition within the intima of apoE3-Leiden mice and elastic fiber thinning and disruption in media layers were highlighted (arrowheads, violet to black staining). Elastic fibers remained intact in apoE3-Leiden : t-PA -/- mice (**L**, thick arrowheads).

Discussion

Although experimental and clinical studies imply a link between disturbances of the fibrinolysis cascade and atherosclerosis, a conclusive conclusion of the plasminogen / plasmin system in atherosclerotic disease has not been established yet.

Our data demonstrate that neither a deficiency in u-PA nor in PAI-1 altered atherosclerosis progression in apoE3-Leiden mice. Deficiency of t-PA combined with the apoE3-Leiden transgene, however, leads to a significant reduction in lesion area. In addition to the effect of the fibrinolytic system on the lesion area of plaques, the effect on the type of lesion was investigated. Our results show that the severity of lesions (type IV and V) is reduced in mice with combined t-PA deficiency and apoE3-Leiden transgene as compared to the control group. The mild lesions (type I-III) are increased in compound mice (apoE3-Leiden : t-PA -/-). These results imply that the mechanism by which t-PA operates in the development of atherosclerosis is most likely related to the accumulation of matrix material, macrophages, and / or smooth muscle cells in the plaque.

It has been shown that t-PA is a potent mitogen for SMCs, suggesting that it may have important functions in atherosclerosis **(44).** It was also suggested that SMCs might regulate their own plasminogen activators in an autocrine fashion **(45).** However, this mitogenic effect was specific for t-PA, since plasminogen, plasmin, and u-PA were not able to support SMC growth. It has been suggested that u-PA and t-PA may have different functions, which emerge during the atherogenesis process **(46).** An increase of t-PA levels was seen at a late stage after the injury of the rat carotid artery, at a time when the SMCs were beginning to migrate from the media to the intima, whereas u-PA accumulates during SMC proliferation (soon after the injury). Indeed, as shown in the present study, deficiency of t-PA combined with the apoE3-Leiden transgene leads to a reduced lesion area and lesion severity, whereas u-PA deficiency did not affect the atherosclerotic process. Traynelis *et al* **(30)** recently showed that t-PA-deficient mice demonstrated approximately 50 % smaller cerebral infarcts as compared to wild-type mice, although there is no relation between our study and the study of Traynelis *et al.* In contrast to our study, Carmeliet *et al* have shown that u-PA-activated plasmin is, through plasmin-activated proMMPs, a risk factor for progression of aneurysm formation **(35).** Possibly this discrepancy relates to

differences in genetic background, the type of diet or the length of the study. We could speculate that u-PA may be involved in other stages of atherosclerosis such as in the very early stages of atherosclerosis (soon after injury) **(44, 46).**

It has also been suggested that t-PA may act as an autocrine factor with two major functions: catalytic activity (conversion of plasminogen into plasmin), and mitogenic activity **(44).** Thus, t-PA may contribute by both means to atherogenesis. The potential mechanisms whereby t-PA deficiency might reduce atherosclerotic lesion formation are through the loss of plasmin-mediated extracellular matrix degradation, the inhibition of plasmin-mediated TGF-β activation in the vessel wall and the reduction of collagen synthesis, the inhibition of plasmin-mediated proMMP activation in the vessel wall and loss of mitogenic effect of t-PA for SMCs. Active TGF-β is a stimulator of the synthesis of extracellular matrix proteins, including collagen. Thus, an impaired t-PA-catalyzed plasmin formation prevents the breakdown of extracellular matrix proteins. This results in the inhibition of cell migration and proliferation and the hampering of the activation and release of latent matrix-bound growth factors such as TGF-β in the matrix. Thus, the absence of plasmin-activated TGF-β in the combined t-PA-deficient and apoE3-Leiden mice may lead to the decrease of collagen synthesis and its deposition in plaques as we have shown. Furthermore, it has recently been shown that an excessive plasmin proteolysis predisposes to tissue destruction **(47).** This finding is reinforced by showing that the loss of growth-arrest-specific gene 6 (GAS-6) results in the accumulation of collagen, disorganizing plaque phenotypes, which in turn leads to intra-plaque bleeding **(48).** Although it has been suggested that the more collagen present in the plaque, the more stable the plaque is, other studies **(47, 48)** do not support this hypothesis. In line with the studies **(47, 48),** Yamamoto *et al* **(15)** recently showed that inhibition of TGF-β resulted in inhibition of neointimal formation, associated by a reduction in collagen synthesis in a rat caritid artery model. Taken together, these results suggest that in mice deficient in t-PA combined with the apoE3-Leiden transgene the accumulation of matrix materials may be decreased and thereby contribute to the disorganization of plaques or delay the progression of atherosclerotic plaques. Our data are consistent with other studies, which suggest that the increase of t-PA is associated with the severity of plaques and thus contributes to the instability of plaques and the risk of rupture **(34).** This finding is reinforced by other observations showing deficiency in PAI-1 did not affect atherosclerosis in apoE3-Leiden mice (present study) as well as the LDL-receptor knockout and apoE-knockout mouse models **(49).** HPS staining

revealed that necrosis which is often accompanied by the infiltration of granulocytes was present in plaques from 4 out of 8 apoE3-Leiden : PAI-1 -/- mice compared to 1 of 5 apoE3-Leiden mice. This finding might indicate that the absence of PAI-1 could result in the necrosis of macrophages, which normally can only be found in severe plaques with apoptosis. The same type of necroses accompanied by the infiltration of granulocytes has been shown in VLDLr -/- : LDLr -/- mice in an early stage of atherosclerotic lesion (type I-III), as compared to control mice (LDLr-/-) (with no incidence of necrosis in the control group). However, this type of necrosis was also observed in advanced lesions of both groups of mice (submitted). These results suggest that PAI-1 could be involved, for example, in the early stages of an atherosclerotic lesion. The nature of the mechanism underlying this increase of necrosis remains to be elucidated.

Our results further demonstrated the presence of SMCs (trace) in the fibrous cap of lesion and media (very high) of apoE3-Leiden mice lacking t-PA, and the virtual absence of such cells in the very advanced lesion of apoE3-Leiden (control group) mice. The latter may be due to SMC transformation in other cell types such as fibroblasts or apoptosis of SMCs. This finding suggests that the growth factor activity of t-PA for SMC may contribute to the acceleration of the atherosclerotic process via the involvement of SMCs proliferation in this process.

In conclusion, our study indicates that t-PA deficiency inhibits the atherosclerotic process. In combined apoE3-Leiden : t-PA -/- mice, the accumulation of matrix materials is possibly decreased, modified, and / or delayed, contributing to an organized plaque structure and a smaller risk of plaque rupture.

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Chapter VII

General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Our investigations reported in this thesis provide new insights into the following issues: the relation between genetic background and basal plasma fibrinogen levels; possible modification of plasma fibrinogen levels via diet, taking into account both the total amount of dietary fat and its composition; the relation between the rate of the fibrinogen response induction and the susceptibility to atherosclerosis; the role of transcription in the regulation of fibrinogen *in vivo*; the role of elevated plasma fibrinogen levels as well as the most important components of plasminogen / plasmin system (PAI-1, u-PA, and t-PA) in the atherosclerotic process.

Cardiovascular disease and atherosclerosis in particular represents the leading cause of disability and mortality in Western countries **(1-5).** There has been much research into potential risk factors for cardiovascular disease and atherosclerosis in particular. Since the epidemiological studies suggest that a high level of plasma fibrinogen may contribute to the severity of atherosclerosis **(6-10),** it is important to gain more knowledge about the regulation of fibrinogen *in vivo*. A better insight into the mechanism of fibrinogen regulation may eventually lead to new strategies for lowering the prevalence of cardiovascular disease.

Studies in humans on the relative contribution of the different factors to atherosclerosis are hampered by the large variation in both environmental and genetic factors and that is why we started studies in mice. Understanding the genetic basis and the environmental factors as well as their interaction in relation to atherosclerosis is very important for designing appropriate preventive measures and therapeutic intervention strategies. For these reasons, in this thesis we designed primarily a coherent, well-established mouse study to elucidate the effect of genetic factors, diets and their interactions on plasma fibrinogen concentrations.

The results described in this thesis show that genetic background determines the basal plasma fibrinogen levels in mice on a regular diet. The consumption of an atherogenic diet alters the plasma fibrinogen levels and the extent of diet-induced changes depends on their genetic background. Human studies regarding the dietary effects on plasma fibrinogen levels gave conflicting results (for reviews see Vorster *et al* **(11)**, and Miller **(12)** and the accompanying references). Our study was performed with genetically homogenous mouse strains and strict diet control, and was not hampered by interindividual variations.

In vitro and *in vivo* studies have shown that the transcriptional process controls the basal and induced expression of the three genes of fibrinogen **(13-16).** One of our observations in this thesis is, that despite the decrease in hepatic fibrinogen Aα-, Bβ-, and γ-chain mRNA levels, the plasma fibrinogen levels significantly increased in all strains in response to semi-synthetic diets. To elucidate whether an acute phase response is involved in the increase observed in plasma fibrinogen levels, we measured two established acute phase markers. Our results demonstrated that these two markers respond to the atherogenic diet, indicating that the diet induced an acute phase response. However, the fibrinogen mRNAs did not respond to this diet. Our result is in contrast to the general hypothesis that an increase in plasma fibrinogen levels is the result of an acute phase response through high fibrinogen mRNAs. Our results are in line with the observations of other investigators showing that several established positive acute phase proteins respond to an acute phase stimulator but fibrinogen does not **(17)** and that the increase in plasma fibrinogen levels in patients with inflammation may occur independently of the acute phase reaction **(18).** These data suggest that the response of fibrinogen as an acute phase marker depends on the type of stimulation.

To date, there are no human and / or mouse studies published regarding the effect of diet on the rate and magnitude of the response of fibrinogen or the relation between the rate of fibrinogen induction and the susceptibility to atherosclerosis. An intriguing observation in this thesis is that genetic background also determines the rate of the fibrinogen response. The diet-induced increase in plasma fibrinogen is very fast in the highly atherosclerosis-susceptible strain and very slow in the highly atherosclerosis-resistant strain. This may indicate that it is the rate of the fibrinogen response and not the basal level, which is linked to atherosclerosis susceptibility.

It has been suggested that synthesis of the Bβ-chain in HepG2 cells is rate-limiting for the assembly and secretion of mature fibrinogen **(19-22).** Therefore, we directed our studies towards the fibrinogen Bβ-gene biosynthesis *in vivo*. We presented evidence that in mice, which overexpress fibrinogen Bβ-chain mRNA level, this neither leads to an increased plasma fibrinogen level nor does it alter the transcription levels of the two other fibrinogen mRNAs (Aα and γ) *in vivo* **(chapter 3).** The latter finding is in line with the observation of Suh *et al* **(23)** that fibrinogen Aα-chain mRNA was not detectable in the liver of homozygous Aα-gene knock-out mice, whereas fibrinogen Bβ-, and γ-chain mRNA levels in knock-out mice were comparable with the wild-type mice.

Note that if the synthesis of Bβ-chain is the rate-limiting step in the assembly and secretion of fibrinogen in mice, we would expect an increase of plasma fibrinogen in β-chain transgenic mice. The mouse fibrinogen Aα-chain knock-out study of Suh *et al* **(23)** suggests that the level of Aα-chain is also not a rate-limiting factor in fibrinogen production. Another explanation for the absence of increased plasma fibrinogen levels in trangenic mice could be due to the fact that there is no co-ordinate overexpression of the Aα and γ genes in mice overexpressing fibrinogen β-gene. These *in vivo* findings are in contrast to *ex vivo* studies (using HepG2 cell lines) that have suggested that the Bβ-chain **(19-22)** or Aα-chain **(24)** are the rate-limiting step in the assembly and secretion of fibrinogen. *In vitro* studies have also suggested that overexpression of any fibrinogen chain mRNA leads to increased synthesis of the other two chain mRNAs resulting in a coordinated elevation in fibrinogen secretion **(19, 25).**

The variation observed in *in vitro* studies could be due to variations in the origin of cell lines and culture conditions. Furthermore, the effect of physiological parameters or other mechanisms may be missed in *in vitro* studies, while this is not the case in *in vivo* studies.

In this thesis, we also presented evidence that mice overexpressing all three genes of fibrinogen have increased plasma fibrinogen levels, pointing to a required co-ordinated overexpression of all three genes **(chapter 4).**

The data from **chapter 2-4** indicate that the regulation of the plasma fibrinogen level is not determined by transcription of the Bβ-chain gene alone, suggesting the involvement of transcription regulation of other fibrinogen genes, post-transcription regulatory mechanisms such as translation, and / or posttranslational modification, assembly of the fibrinogen from the chains, secretion and degradation **(26)**, clearance, or interplay between these processes.

To elucidate the role of high plasma fibrinogen levels (risk marker or risk factor) in the atherosclerotic process, we crossbred a well-established model for atherosclerosis, apoE3-Leiden transgenic mice **(27),** with mice overexpressing fibrinogen (+/-). The genetic compound offspring were used to study diet-induced atherosclerosis.

Our data **(chapter 5)** demonstrated that there are no differences between apoE3-Leiden : fibrinogen overexpression (+/-) transgene with respect to lesion area as well as lesion composition as compared to apoE3-Leiden transgenic mice after the mice have been fed the atherogenic diet. These results imply that an elevated plasma fibrinogen level does not alter the progression of atherosclerotic lesions in apoE3-

Leiden transgenic mice. Consistent with our results, it has been shown that compound mice (apoE -/- : fibrinogen knock-out (-/-)) do not show any difference in the development of atherosclerotic plaques as compared to apoE -/- transgenic mice **(23).** Indeed we observed no lesion formation in mice overexpressing fibrinogen (+/-) after consumption of the atherogenic diet. Taken together, these results suggest that a combination of the apoE3-Leiden transgene and an atherogenic diet is necessary to induce atherosclerosis and the progression of atherosclerosis is independent of an elevated plasma fibrinogen level in this model.

In none of the mice studied did we observe a thrombus in the atherosclerotic plaques, nor did we observe any fibrin deposition in the complicated lesions of apoE3-Leiden transgenic mice deficient in PAI-1, u-PA, or t-PA **(chapter 6).** The presence of extensive fibrin depositions in most complex lesions in the human situation **(28, 29)** suggests that the occurrence of thrombosis is a critical component in the pathogenesis of human atherosclerosis and is in contrast to the atherosclerosis as observed in mouse models. To date, all generated transgenic mouse models developed to study atherosclerosis lack the thrombus component, which is an important feature of the complicated plaques typically observed in humans. In addition, neither stroke nor myocardial infarction as a result of the vascular occlusion, characteristic of the disease in man, has as yet been shown in the mouse **(30, 31).** The currently available transgenic mouse models are not appropriate for studying the role of fibrinogen in atherosclerosis (apoE-deficient, LDL-receptor knock-out, **(30-32)** and apoE3-Leiden mice) , since the complicated plaques lack the thrombus in these models. The role of hyperfibrinogenemia in complicated plaques with a thrombotic component associated with stroke or myocardial infarction in humans cannot be excluded. Although experimental and clinical studies imply a relation between disturbances of the fibrinolysis cascade and atherosclerosis, a causal relation between the plasminogen / plasmin system and atherosclerotic disease has not been established.

Our results **(chapter 6)** showed that neither a deficiency in u-PA nor in PAI-1 modifies atherosclerosis progression in apoE3-Leiden mice. Deficiency of t-PA combined with the apoE3-Leiden transgene, however, results in a significant reduction in lesion area. We further showed that the severity of lesions (type IV and V) are reduced and the mild lesions (type I-III) are increased in mice with combined t-PA deficiency and apoE3-Leiden transgene as compared to the control group. An important step in the process of plaque formation is the proliferation and migration of smooth muscle cells (SMCs) from the media to the intima. It has been shown that t-

PA is a potent mitogen for SMCs, suggesting that it may have important functions in atherosclerosis **(33).** It was also suggested that SMCs might regulate their own plasminogen activators in an autocrine fashion **(34).** This mitogenic effect was specific to t-PA, since plasminogen, plasmin, and u-PA were not able to support SMC growth. It has been suggested that u-PA and t-PA may have different functions, which emerge during the atherogenesis process **(33, 35).** An increase in t-PA levels was seen at a late stage after the injury of the rat carotid artery, at a time when the SMCs were starting to migrate from the media to the intima, whereas u-PA accumulates during SMC proliferation (soon after the injury) **(34).** Indeed, as shown in the present study deficiency of t-PA combined with the apoE3-Leiden transgene leads to a reduced lesion area and lesion severity, whereas u-PA deficiency does not affect the atherosclerotic process. We could speculate that u-PA may be involved in other stages of atherosclerosis such as in the early stages of atherosclerosis (soon after injury) **(35).**

It has also been suggested that t-PA may act as an autocrine factor with two major functions: catalytic activity (conversion of plasminogen into plasmin), and mitogenic activity **(33).** The potential mechanisms whereby t-PA deficiency might reduce atherosclerotic plaque formation are through the loss of plasmin-mediated extracellular matrix degradation, the inhibition of plasmin-mediated proMMP activation, the inhibition of plasmin-mediated TGF-β activation in the vessel wall and therefore also the reduction of collagen synthesis, and the loss of mitogenic effect of t-PA for SMCs. Active TGF-β is a stimulator of the synthesis of extracellular matrix proteins, including collagen **(36, 37).** Thus, an impaired t-PA-catalyzed plasmin formation would prevent the breakdown of extracellular matrix proteins, resulting in the inhibition of cell migration and proliferation and hampering the activation and release of latent matrix-bound growth factors such as TGF-β in the matrix. Thus, the absence of plasmin-activated TGF-β in the combined t-PA-deficient and apoE3-Leiden mice may lead to the decrease of collagen synthesis and its deposition in plaques as we have shown. Our result is in line with the results of Lutgens *et al* **(38)** demonstrating that an excessive plasmin proteolysis predisposes to tissue destruction. It has also been shown that the loss of growth-arrest-specific gene 6 (GAS-6) results in the accumulation of collagen, disorganizing plaque phenotypes, leading to intra-plaque bleeding **(39).** Although it has been suggested that the more collagen present in the plaque, the more resistant to rupture the plaque is, other studies **(38, 39)** do not support this hypothesis. Our data further suggest that the growth factor activity of t-

PA for SMC may contribute to acceleration of the atherosclerotic process via the involvement of SMC proliferation in this process. The increase of t-PA may also contribute to acceleration of the atherosclerotic process via the involvement of SMC migration.

Our results show that in the t-PA knock-out mice combined with the apoE3-Leiden transgene a decrease in the accumulation of matrix materials occurs, which could contribute to the disorganization of plaque structure or delay the progression of atherosclerotic plaques. Our data are consistent with other studies, which suggest that the increase in t-PA is associated with the severity of plaques and thus contributes to the instability of plaques and the risk of rupture **(40).** The results observed regarding the association between increase in t-PA and the severity of atherosclerose are supported by other observations showing that the deficiency of PAI-1 does not affect atherosclerosis in apoE3-Leiden mice (present study) nor in LDL-receptor knockout and apoE knockout mice **(32).**

Conclusion

From the observations in this thesis and the studies of other investigators, the role of fibrinogen and fibrinolytic system in the atherosclerotic process seems to be very complex.

The results described in this study do not show a causal relationship between fibrinogen, u-PA, or PAI-1 and the atherosclerotic process.

On the bases of the obtained data, it might be concluded that it is the rate of the response of fibrinogen to diet rather than the actual level, which relates to atherosclerosis susceptibility.

In this investigation, however, a causal relationship between t-PA and the severity of the atherosclerosis is established. This could be due to the role of t-PA in the organization of plaque via cell proliferation and migration, and the degradation of extracellular matrix materials. This study can lead to additional preventive and therapeutic intervention strategies for cardiovascular disease and atherosclerosis in particular.

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Chapter VIII

Summary

SUMMARY

In **chapter 1**, fibrinogen, tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and plasminogen activator inhibitor-1 (PAI-1), key components of coagulation and the plasminogen/plasmin system, respectively, and atherosclerosis are introduced.

Cardiovascular disease and atherosclerosis in particular is the leading cause of morbidity and mortality in Western countries. Atherosclerosis is a disease of multifactorial etiology involving the interaction between genetic and environmental factors modulating the functions of various cell types and inflammatory molecules within the vessel wall. The vascular wall, lipoprotein-metabolism, coagulation cascade, and fibrinolytic system play a central role in atherosclerosis.

The haemostatic mechanism can be characterized as a dynamic balance between two opposing processes, fibrin formation (coagulation) and fibrin degradation (fibrinolysis). The former ensures that after tissue damage a fibrin clot is formed, which prevents bleeding, whereas, the latter guarantees tissue repair by the removal of fibrin clots. They also form part of a wide range of pathological and physiological processes and are known as important elements of atherogenesis and atherothrombosis.

Although from many epidemiological studies it is apparent that elevated plasma fibrinogen concentrations form one of the most important independent risk factors in blood for cardiovascular disease and particularly atherosclerosis in humans, they do not prove a causal relationship. This uncertainty is enhanced by the fact that fibrinogen is an acute phase protein with concentrations rising in response to inflammation. Since atherosclerosis itself has an inflammatory component, it is believed that plasma fibrinogen concentrations may reflect the severity of atherosclerosis. Data from the literature suggest that a critical role exists for inflammation in both atherogenesis and acute thrombosis. In this thesis, we investigated whether fibrinogen may be considered as a simple marker or a real cause of atherosclerosis.

Although epidemiological, genetic, clinical and molecular evidence suggests that the plasminogen / plasmin cascade may be involved in vascular disease and particularly atherosclerosis, this association is not yet proven. The plasminogen / plasmin activation cascade is involved in extracellular matrix proteolysis, cell migration and proliferation, tumor cell invasion and metastic spread, activation of latent growth

factors as well as zymogens of the matrix-metalloproteinase enzymes, and generation of biologically active fibrin degradation products (FDPs). Thus, plasmin-mediated proteolysis (and / or activation) could be involved in the progression of atherosclerotic lesions through several distinct mechanisms. In this thesis, we also studied whether the plasminogen activators (t-PA and u-PA) and plasminogen activator inhibitor-1 (PAI-1) are involved in the progression of the atherosclerotic lesion and at what level of plaque formation this occurs.

Heterogeneity in both genetic and environmental factors as well as their interaction hampers the identification of the individual genetic and environmental factors involved in the development of atherosclerosis in humans. To study the effect of an elevated fibrinogen in plasma, t-PA, u-PA, and PAI-1 on the development of atherosclerosis in a suitable animal model, requires the study of these factors under well-defined genetic and well-controlled environmental conditions. To achieve this, we used two recently developed technologies, gene targeting (homologous recombination in embryonic stem (ES) cells) and transgenesis (gene transfer). this allowed us to manipulate the genetic balance of candidate molecules in mice in a controllable manner. Thus, transgenic mice models have proven to be useful tools for studying many aspects of human cardiovascular disease in a homogenous genetic and environmental background. To prevent the above-mentioned limitations, we performed our studies in the transgenic mouse models. We chose apoE3-Leiden transgenic mice as a model for assessing atherosclerosis, because the lipoprotein profiles in these mice are very highly comparable to the ones found in humans. Furthermore, plaque formation could be induced, modulated and measured reproducibly in these mice by variation of the diet, as in the human situation. This mouse model was used as genetic background for mice deficient in t-PA, u-PA, PAI-1, and mice with an increased level of fibrinogen in plasma.

Understanding the genetic basis and the environmental factors as well as their interaction in relation to atherosclerosis is very important for designing appropriate preventive measures and therapeutic intervention strategies. In this thesis we designed primarily a mouse study to clarify the effect of genetic factors, diets and their interactions on plasma fibrinogen concentrations. We examined plasma fibrinogen levels in four strains of mice, which differ in their susceptibility to cholesterol-induced atherosclerosis. The mice were fed normal breeding chow and four different semi-synthetic diets for different time intervals **(chapter 2).** When maintained on basal diet, two strains 129/J and C3H/HeJ demonstrated a significantly higher plasma

fibrinogen concentration than C57BL/6J and BALB/C strains. The strongest and most rapid increase of plasma fibrinogen (by all diets) as well as the acute phase marker haptoglobin (only by atherogenic diet) is observed in C57BL/6J mice, which are known to be highly susceptible to diet-induced atherosclerosis.

An increase in plasma fibrinogen was observed in all strains on semi-synthetic diets. No increase was observed in the fibrinogen Aα- Bβ- and γ-chain mRNA levels in the liver on the same diets. The fibrinogen mRNA levels were even decreased in mice on the atherogenic diet.

This study indicates that: genetic background determines the plasma fibrinogen levels on basal diet; diet alters plasma fibrinogen levels, and the extent of the changes due to diet depends on the genetic background; the increase of fibrinogen in plasma due to the diets is independent of transcription; the diet-induced increase of fibrinogen was very fast and strong in the very high atherosclerosis-susceptible strain C57BL/6J and very slow and weak in the very high atherosclerosis-resistant strain C3H/HeJ. It might be concluded that it is the kinetics of the response of fibrinogen on diet rather than the actual level, which relates to atherosclerosis susceptibility.

Since the epidemiological studies suggest that a high level of plasma fibrinogen may play a role in the severity of the atherosclerosis, it is important to gain more knowledge about the regulation of fibrinogen. We have therefore undertaken an *in vivo* study of fibrinogen biosynthesis in the mouse. Moreover, it has been suggested that synthesis of the Bβ-chain is rate-limiting for the assembly and secretion of mature fibrinogen. Therefore, we directed our studies toward the fibrinogen β-gene biosynthesis *in vivo*. We generated and characterized transgenic mice with overexpression of the fibrinogen β-gene **(chapter 3).** Southern blot analysis identified a founder that carried additional copies of the fibrinogen Bβ-chain gene. Northern blot analysis demonstrated approximately a 3-fold increase in fibrinogen Bβ mRNA in heterozygous mice as compared to wild-type, whereas homozygous transgenic mice showed approximately a 9-fold increase. The levels of the Aα and γ mRNAs in transgenic homozygous mice were not altered as compared to those in wild-type mice. Fibrinogen levels in plasma were not significantly increased in transgenic mice as compared to wild-type-mice.

These results indicate that: additional copies of the fibrinogen Bβ-chain gene lead to increased levels of the Bβ-chain mRNA in the liver; the increased levels of Bβ-chain mRNA in homozygous overexpression mice do not change the transcription levels of

the two other fibrinogen mRNAs *in vivo*; the absence of an increased plasma fibrinogen level in the transgenic mice indicates that this level is not regulated solely by transcription of the Bβ-chain gene.

Although increased plasma fibrinogen levels are linked with the severity of the atherosclerosis as shown by many epidemiological studies, they do not establish which comes first. Because the epidemiological studies are not able to answer this question unambiguously, a mouse model of hyperfibrinogenemia was as a first step generated by conventional transgenesis to distinguish these two possibilities. **Chapter 4** describes the generation and characterization of transgenic mice with overexpression of all three genes of fibrinogen. One transgenic line was identified with elevated fibrinogen (1.7-fold relative to wild-type mice). Northern blot analysis demonstrated that transgene expression was liver-specific and fibrinogen Aα, Bβ, and γ mRNA levels were 2-3-fold enhanced.

Our data indicate that a co-ordinated overexpression of all three fibrinogen genes in mice leads to hyperfibrinogenemia.

Transgenic mice with hyperfibrinogenemia were crossbred with atherosclerosis-prone mice, apoE3-leiden mice, and genetic compound was used to study the contribution of fibrinogen in the development of atherosclerosis after the mice were fed an atherogenic diet **(chapter 5).**

The lesion area of plaques in the aortic valve was not significantly different in apoE3-Leiden : fibrinogen overexpression (+/-) mice as compared to apoE3-Leiden transgenic mice. No thrombus was observed in atherosclerotic lesions of either group of mice.

These results indicate that elevated plasma fibrinogen levels in apoE3-Leiden do not affect the progression of atherogenic diet-induced atherosclerosis.

To explore whether variations in endogenous fibrinolytic activity alter the process of atherosclerosis, we investigated the effect of genetic modification of PAI-1, u-PA, and t-PA expression in apoE3-Leiden transgenic mice. These atherosclerosis-prone mice were cross-bred with PAI-1 -/-, u-PA -/-, and t-PA -/-, and genetic compound offspring evaluated for atherosclerosis progression on a mild atherogenic diet **(chapter 6).** The lesion area of plaques in the aortic valve was not significantly different in apoE3-Leiden: PAI-1 -/- and apoE3-Leiden: u-PA -/- mice as compared to apoE3-Leiden mice. In contrast, the absence of t-PA (-/-) in apoE3-Leiden transgenic mice resulted in a significant 70 % reduction of the lesion area as compared to control group apoE3-

Leiden transgene. In addition the early, regular fatty streaks and mild plaques increased in apoE3-Leiden : t-PA -/- mice, whereas the severe plaques (type IV and V) decreased in the combined genotype group (apoE3-Leiden : t-PA -/-).
Higher deposition of collagen was observed in the atherosclerotic lesions of apoE3-Leiden mice as compared to apoE3-Leiden : t-PA -/- mice.
Our results indicate that t-PA deficiency reduces the atherosclerotic plaque formation in this mouse model. The mechanisms whereby t-PA deficiency delays the atherosclerotic process are suggested to be the loss of plasmin-mediated extracellular matrix degradation, the inhibition of plasmin-mediated TGF-β activation and zymogen matrix metalloproteinase (MMP) activation in the vessel wall (particularly decrease of collagen synthesis), and the loss of mitogenic activity of t-PA on SMCs.
In conclusion, the study performed in this thesis does not demonstrate a causal relationship between fibrinogen, u-PA as well as PAI-1 and atherosclerosis. However, a causal relationship between t-PA and the severity of the atherosclerosis is clearly established. This study can open up the way for additional preventive and therapeutic intervention possibilities for cardiovascular disease and atherosclerosis in particular.

Chapter IX

Samenvatting

SAMENVATTING

Hart- en vaatziekten, vooral atherosclerose, zijn al jaren de belangrijkste doodsoorzaak in de westerse wereld. Met name het hart- en herseninfarct zijn plotselinge, ernstige gevolgen van de langzaam optredende vernauwingen (atherosclerose) in de bloedvaten. Het woord atherosclerose is afgeleid van de griekse woorden voor vettigheid (atheros) en verharding (scleros). Bij de meeste hartpatiënten is sprake van ziekte van de kransvaten: er vindt vetafzetting plaats in de wanden van de kransslagaders, die de hartspier van bloed voorzien. Die vetafzetting leidt tot vernauwing. Als een kransslagader afsluit, heeft de hartspier plaatselijk geen bloedaanvoer meer en sterft af. Atherosclerose is een ingewikkeld proces waarbij veel factoren zijn betrokken. Op grond van de huidige inzichten lijken het lipoproteine metabolisme, de bloedstolling en fibrinolyse en de vaatwand de belangrijkste betrokken componenten te zijn.

In diverse epidemiologische onderzoeken bleken verhoogd VLDL/LDL en fibrinogeen de twee meest opvallende onafhankelijke risicofactoren te zijn voor hart- en vaatziekten. Een oorzakelijk verband tussen verhoogd VLDL/LDL en atherosclerose is overtuigend aangetoond, maar voor fibrinogeen is dit niet het geval. Hoewel de precieze moleculaire mechanismen betrokken bij het ontstaan en voortschrijden van atherosclerose nog verre van opgehelderd zijn, wordt algemeen gedacht dat groei en migratie van gladde spiercellen noodzakelijk is voor de ontwikkeling van een atherosclerotische plaque. In vitro experimenten suggereren dat de plasminogeen activatoren t-PA en u-PA een rol spelen bij de migratie van gladde spiercellen.

In het hier uitgevoerde onderzoek hebben wij onderzocht of in een muismodel voor atherosclerose aanwijzingen konden worden gevonden voor: - een oorzakelijke relatie tussen fibrinogeen en atherosclerose vorming; - een in vivo betrokkenheid van de plasminogeen activatoren u-PA en t-PA en de remmer van plasminogeen activatoren PAI-1 bij gladde spiercelmigratie en vorming van atherosclerotische plaques.

De plasma spiegels van fibrinogeen van verschillende muizenstammen bleken flinke verschillen te vertonen. Onverwacht bleek het plasma fibrinogeen niveau sterk afhankelijk van het dieet, waarbij de reactie op het dieet weer stam-afhankelijk bleek te zijn.

Het mechanisme achter deze plasma fibrinogeen verhoging is niet, zoals in eerste instantie verwacht, terug te voeren op een verhoogde genexpressie van één of meer van de fibrinogeen genen. Ook konden wij geen relatie ontdekken tussen de respons van plasma fibrinogeen op het dieet en de respons van andere acute fase eiwitten, hetgeen suggereert dat de stijging van fibrinogeen niet zomaar berust op een generieke respons op ontsteking.

In deze proefdieren is het niet zozeer het plasma fibrinogeen als zodanig dat correleert met de atherosclerose gevoeligheid, maar is er wel een verband tussen de snelheid van de fibrinogeen stijging na dieet verandering en de atherosclerose gevoeligheid van de muizenstam. Zo bleek fibrinogeen zeer snel te stijgen in de atherosclerose gevoelige stam C57BL/6J terwijl een langzame stijging werd waargenomen in de atherosclerose resistente stam C3H/HeJ. De uiteindelijke plasma fibrinogeen spiegels waren echter niet significant verschillend.

Omdat literatuur data suggereerden dat van de drie verschillende fibrinogeen ketens (Aα, Bβ en γ) de expressie van de Bβ keten snelheidsbepalend was voor de synthese van fibrinogeen in celcultures zijn wij gestart met het construeren van een transgene muis met extra exemplaren van het Bβ gen.

In deze transgene muis bleek zoals verwacht een verhoging van het Bβ mRNA te worden gevonden gecorreleerd aan het aanwezig zijn van extra gen kopieën. Merkwaardig genoeg werd geen signifcante stijging van het plasma fibrinogeen niveau gemeten.

Onze conclusie is dat in vivo de regulatie van de fibrinogeen synthese aanzienlijk complexer blijkt te zijn dan in celcultures. Voor onze verdere in vivo experimenten hebben wij dan ook transgene muizen gebruikt die extra exemplaren van zowel de Aα, Bβ als γ fibrinogeen genen bezitten. Transgene muizen met overexpressie van alle drie de genen van fibrinogeen (Aα, Bβ, en γ) blijken wel een verhoogde plasma fibrinogeen spiegel te hebben.

Door kruising van transgene muizen met verhoogd plasma fibrinogeen met atherosclerose gevoelige muizen met het apoE3-Leiden transgen zijn muizen verkregen met beide eigenschappen. Deze dieren die zowel verhoogd plasma fibrinogeen als atherosclerose gevoeligheid vertonen zijn vervolgens gebruikt voor onze experimenten. Onze experimenten hebben geen relatie kunnen aantonen tussen plasma fibrinogeen spiegels en atherosclerose gevoeligheid in deze muis modellen.

De bijdrage van het fibrinolytische systeem in atherosclerose vorming in vivo is bestudeerd met behulp van muizen die enerzijds gevoelig gemaakt zijn voor atherosclerose door de inbouw van een humaan apoE3-Leiden gen en anderzijds telkens één van de sleutelcomponenten van het fibrinolytische systeem missen door specifieke inactivatie van het bewuste fibrinolyse systeem gen. Deze combinatie muizen zijn verkregen door kruising van apoE3-Leiden transgene muizen met muizen met een geïnactiveerd u-PA, t-PA of PAI-1 gen.

De atherosclerose ontwikkeling bleek niet te verschillen in de apoE3-Leiden muizen en de apoE3-Leiden transgene muizen die ofwel u-PA, ofwel PAI-1 deficiënt waren. Combinatie van het apoE3-Leiden transgen met t-PA deficiëntie leidt echter tot een zeer significante reductie in de grootte van de atherosclerotische plaques. Bovendien bleek er een aanzienlijke verschuiving van ernstige naar minder ernstige plaques te hebben plaats gevonden. De plaques in de muizen met gecombineerde t-PA deficientie en aanwezigheid van het apoE-3Leiden gen bevatten duidelijk minder collageen dan de plaques in muizen met alleen het apoE3-Leiden gen.

Het mechanisme achter deze waarnemingen is nog niet geheel duidelijk. Uit de literatuur en onderzoek in ons eigen laboratorium is n.l. gebleken dat t-PA betrokken kan zijn bij diverse stappen in het atherosclerose proces: - t-PA kan de groei van gladde spiercellen bevorderen; - t-PA kan de migratie van gladde spiercellen stimuleren; - t-PA kan het oplossen van fibrine in de plaque en daarmee de plaque structuur beïnvloeden en t-PA kan de activatie van groeifactoren zoals TGF-β reguleren. Het relatieve belang van deze mogelijke mechanismen bij de atherosclerose vorming is nog niet duidelijk.

Uit het hier beschreven onderzoek en op andere plaatsen uitgevoerd recent onderzoek blijkt dat de rol van fibrinogeen en het fibrinolytische systeem in atherosclerose vorming complex is. Een direct oorzakelijk verband tussen fibrinogeen plasma spiegels en atherosclerose risico is nog steeds niet echt duidelijk. Uit onze resultaten zou voorzichtig kunnen worden opgemaakt dat wellicht niet het eigenlijke fibrinogeen niveau, maar veeleer de reactie van het fibrinogeen niveau op een prikkel van buiten het meest belangrijk is.

Het fibrinolytisch enzym t-PA blijkt zeer duidelijk betrokken bij het atherosclerose proces. Het meest waarschijnlijk is een rol bij de organisatie van de plaque door de stimulatie van celgroei en migratie en degradatie van extracellulaire matrix eiwitten.

Hoewel de huidige resultaten met enige voorzichtigheid moeten worden geïnterpreteerd lijkt het plasminogeen activatie systeem een interessant doelwit voor evt. therapeutische interventie in het atherosclerose proces mogelijk als aanvulling op maatregelen of therapie gericht op het beïnvloeden van lipiden en/of lipoproteinen.

Atherosclerose of (slag) aderverkalking is een belangrijke oorzaak van hartinfarcten en andere vaataandoeningen. Vetten, waaronder cholesterol zijn betrokken bij het ontstaan van atherosclerose. Verlaging van plasma cholesterol d.m.v. dieet of medicatie heeft een remmende effect op het ontstaan en voortschrijden van atherosclerose. In ons onderzoek hebben wij in dierexperimenten het verband onderzocht tussen factoren betrokken bij het ontstaan en weer oplossen van bloedstolsels en het atherosclerose proces. Wij hebben geen oorzakelijk verband kunnen aantonen tussen de stollingsfactor fibrinogeen en atherosclerose, hoewel er wel een verband wordt vermoed uit bevolkingsonderzoek. Er is wel een duidelijke relatie gevonden tussen het ontstaan en de ernst van atherosclerose en de bloedstolsel oplossende factor t-PA. Dit onderzoek kan leiden tot nieuwe additionele therapeutische mogelijkheden als aanvulling op de thans gangbare cholesterol verlagers.

ABBREVIATION

ANOVA	analysis of variance
Apo-B	apolipoprotein B
APOE	apolipoprotein E
Bp	basepair
β-VLDL	β-very low density lipoprotein
C	control diet (chow)
cDNA	complementary DNA
CHD	coronary heart disease
CVD	cardiovascular disease
DSP	diagnostic set primer
DNA	deoxyribonucleic acid
ECM	extracelular matrix
EL	elastin
ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem
Fbg	fibrinogen
FDPs	fibrin degradation products
FT+	fibrinogen transgene positive
FT-	fibrinogen transgene negative
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS-6	growth-arrest-specific-6
GF	growth factor
HDL	high density lipoprotein
HPS	hematoxylin-phloxin-saffron
Kb	kilobase pair
KDa	kilo dalton
LDL	low density lipoprotein
LDL-C	low density lipoprotein-cholesterol
HMW	high molecular weight
LMW	low molecular weight
LMW'	low molecular weight'
LDL-r	low density lipoprotein-receptor
Mφ	macrophage

MMP	matrix metalloproteinase
MMUP	mouse major urinary protein
MSB	matrius-scarlet-blue
N	atherogenic diet
Ovex	overexpression
P	mild atherogenic diet
P1	phage 1 library
PAI	plasminogen activator inhibitor
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffer saline
PBST	phosphate buffer saline tween 20
PCR	polymerase chain reaction
PCHO	plasma cholesterol
Pls-bkg	pulse minus background
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SMC	smooth muscle cell
SR	sirius red
T	saturated fat diet
TBST	tris buffered saline tween 20
TGF-β	transforming growth factor-β
TMB	tetramethylbenzidine
t-PA	tissue-type plasminogen activator
T3F	T3 forward
T3R	T3 reverse
T7F	T7 forward
T7R	T7 reverse
VLDL	very low density lipoprotein
VLDL-r	very low density lipoprotein-receptor
u-PA	urokinase-type plasminogen activator
UV	ultraviolet
X	unsaturated fat diet
Z	sucrose-rich diet

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Rezaee F, Gulledge A, Gijbels M, Offerman E, van der Linden M, Lord S, Verheijen JH Overexpression of fibrinogen in apoE3-Leiden transgenic mice does not influence the progression of cholesterol-induced atherosclerosis. **Submitted**

Rezaee F, Gijbels MJ, Offerman EH, Verheijen JH. Genetic deletion of tissue-type plasminogen activator (t-PA) in apoE3-Leiden mice reduces progression of cholesterol-induced atherosclerosis. **Submitted**

Curriculum Vitae

Farhad Rezaee werd geboren op 20 oktober 1960 in Iran, Teheran. In 1979 behaalde hij zijn VWO diploma (experimentele biologie) aan het Dehgodah College te Karaj. In 1990 begon hij de studie medische biologie aan de hogeschool Alkmaar. Hiervoor werd in 1994 het diploma behaald. In datzelfde jaar begon hij de studie medische biologie aan de Vrije Universiteit Amsterdam. Hiervoor werd in 1994 de propedeuse behaald. Het doctoraalexamen werd in augustus 1995 behaald met als specialisatie moleculaire biologie. Vanaf oktober tot en met januari (1995-1996) was hij werkzaam bij de afdeling experimentele endocrinologie en oncologie van de Vrije Universiteit te Amsterdam. Van maart 1996 tot maart 2000 was hij werkzaam als assistent in opleiding aan het Leids Universitair Medisch Centrum, afdeling Inwendige Geneeskunde, gedetacheerd bij het Gaubius Laboratorium, TNO-PG te Leiden. Daar werd het in dit proefschrift beschreven onderzoek met een subsidie van de Nederlandse Hartstichting uitgevoerd. Vanaf februari 2001 is hij aangesteld als postdoctoraal onderzoeker aan de afdeling Metabole Ziektes, en Gastro-enterologie, Universitair Medisch Centrum Utrecht (WKZ). Hier wordt onderzoek gedaan naar het mechanisme van het FIC-1 gen in progressive familiar intrahepatic cholestasis (PFIC).