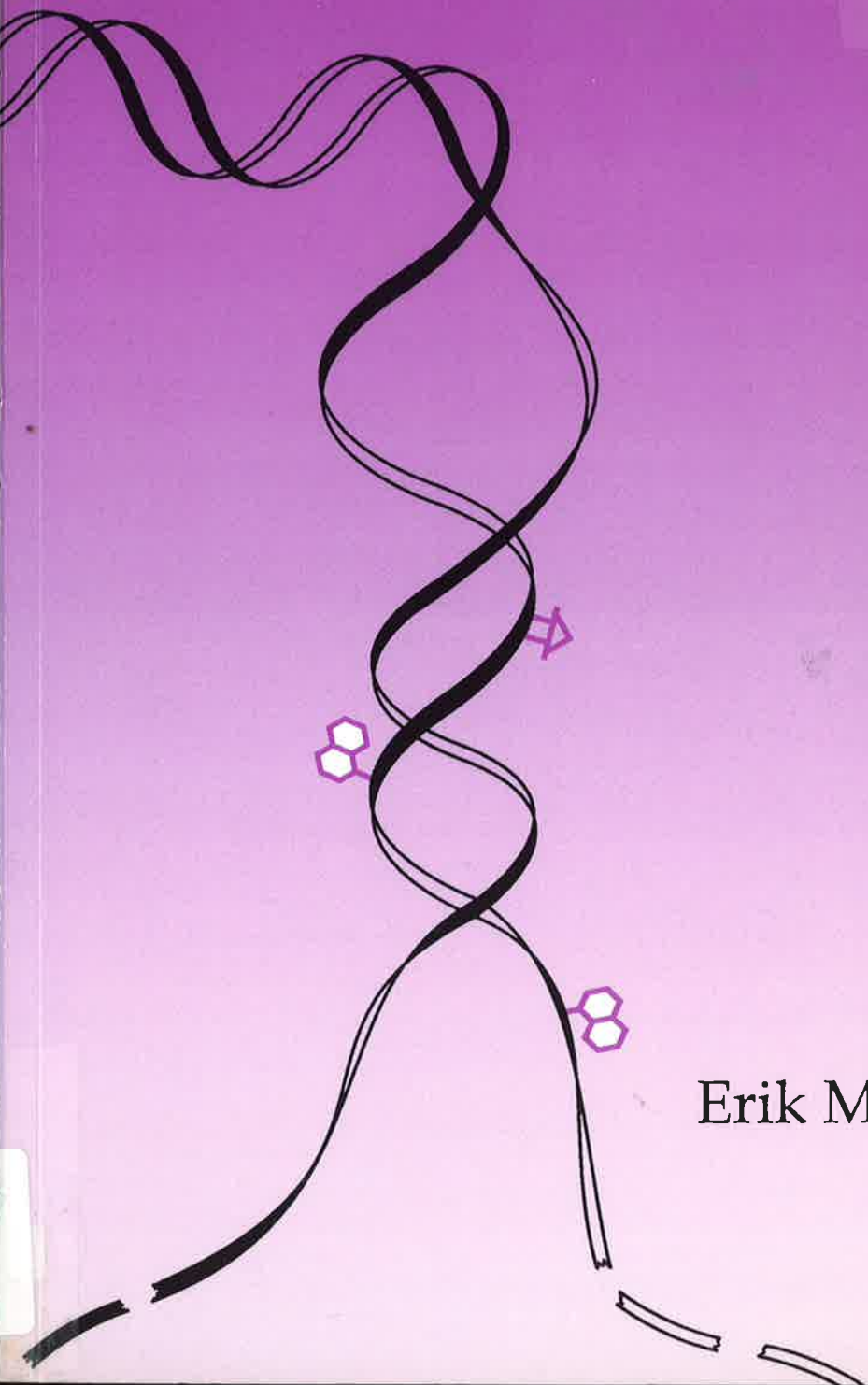
DNA DAMAGE AND REPA
IN RELATION TO AGING



Erik Mullaart

DNA DAMAGE AND REPAIR IN RELATION TO AGING

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STELLINGEN

1

Aangezien Niedermüller bij zijn experimenten hoge concentraties van DNA beschadigende agentia heeft gebruikt, is zijn conclusie dat het vermogen om bepaalde DNA-schades te herstellen afneemt met de leeftijd voorbarig.

Niedermüller, H. (1982) Mech. Ageing Dev., 19, 259-271.

2

Gezien het lage detectieniveau van de door Su et al. gebruikte methode voor het meten van DNA-breuken is het onmogelijk te concluderen dat het aantal breuken in de hersenen van muizen gelijk blijft tijdens het ouder worden.

Su, C.M. et al. (1984) Mech. Ageing Dev., 27, 239-247.

3

De extrapolatie van DNA-herstelwaarden gemeten in vitro naar de situatie in vivo heeft geen waarde omdat het herstel van door UV licht-geïnduceerde DNA-schades in gekweekte rattehuidcellen veel lager is dan in de zelfde cellen in vivo.

Dit proefschrift.

4

De observatie dat benzo(a)pyrene in rattelever weinig of geen DNA-adducten maar voornamelijk DNA-breuken induceert, kan verklaren waarom dit agens in dit dier geen levertumoren veroorzaakt.

Leadon et al. (1988) Proc. Natl. Acad. Sci. USA., 85, 4365-4368.

Dit proefschrift.

5

Wanneer de DNA-herstelsystemen DNA-schades in de hersenen vergeten te herstellen, kan vergeetachtigheid het gevolg zijn.

Dit proefschrift.

6

De door Swaab et al. gevonden activatie van bepaalde hersencellen wijst erop dat hersenveroudering niet altijd gepaard hoeft te gaan met achteruitgang van celfuncties.

Swaab et al., (1987) Eur. J. Cell Biol., 44, 27-28.

7

Bij de ontwikkeling van "gezonde" leef- en eetgewoonten met het doel langer te leven dient men zich te realiseren dat veel DNA-schades veroorzaakt worden door lichaamswarmte en zuurstof.

8

Hoewel Down's syndroom gekarakteriseerd wordt door een trisomie van chromosoom 21, is het zuiver toeval dat er een genetische koppeling is van de ziekte van Alzheimer met een gebied op dit zelfde chromosoom.

9

Het hoge percentage (± 20 %) publicaties van Engelse groepen in Nature zegt niets over de kwaliteit van het wetenschappelijk onderzoek in het Verenigd Koninkrijk.

10

De huidige vergrijzing, in combinatie met de bezuinigingen op de kosten van de verzorging van bejaarden, maken een versnelde oplossing van verouderings-gerelateerde ziekten, zoals de ziekte van Alzheimer, noodzakelijk.

11

Onderzoek naar veroudering is een kwestie van leven en dood.

12

Wie glimlacht is sterker dan wie raast.

Stellingen behorende bij het proefschrift
"DNA damage and repair in relation to aging"
E. Mullaart
13 september 1989

DNA DAMAGE AND REPAIR IN RELATION TO AGING

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. J.J.M. BEENAKKER, HOOGLERAAR IN DE FACULTEIT DER WISKUNDE EN NATUURWETENSCHAPPEN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 13 SEPTEMBER 1989 TE KLOKKE 15.15 UUR

DOOR

ERIK MULLAART

geboren te Rotterdam in 1959

1989

krips repro meppel

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The work described in this thesis was performed at the TNO institute for Experimental Gerontology and the Medical Biological Laboratory of the TNO Division of Health Research.

Aan mijn ouders
Voor Hanneke

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CHAPTER 1

GENERAL INTRODUCTION
DNA DAMAGE METABOLISM AND AGING

1.1 Introduction

All living organisms are continuously exposed - to various extents - to agents that can damage their DNA, RNA and proteins. Especially damage to the DNA, the carrier of the genetic information, can have serious consequences for the organism. The maintenance of DNA integrity in somatic cells over long time periods, therefore, is of paramount importance. The possibility that gradually imperfections are introduced in the somatic DNA is a central element in several aging theories (Hart et al., 1979; Hayflick, 1980; Gensler and Bernstein, 1981; Vijg, 1987).

If not repaired, DNA damage can lead to dysfunctioning of cell and tissue and it might well be one of the underlying causes of the age-related reduction in homeostatic capacity and the increased incidence of cancer and other diseases of old age such as Alzheimer's disease. To overcome the harmful effects of DNA damage, the cell has several DNA-repair systems, which are able to remove or circumvent lesions that might otherwise interfere with DNA replication and transcription. However, since DNA repair is not always perfect, it is not inconceivable that certain forms of DNA damage accumulate with age; age-related decreases in DNA-repair efficiency would enhance such an effect. Inter-individual differences in the extent of exposure to DNA-damaging agents but also in the activity of DNA-repair systems can lead to large inter-individual differences in the rate of damage accumulation. This might explain the variation observed in the aging rate among human individuals.

In the studies that form the basis of this thesis, the hypothesis that DNA-repair activities decline during aging was experimentally tested by investigating

the induction and removal of specific DNA lesions in skin and liver cells from young and old rats. In addition, the possibility that DNA damage accumulates during aging was directly tested by assessing basic levels of DNA damage in liver and brain of young and old rats. To test the hypothesis that a defective DNA damage metabolism is involved in the etiology of Alzheimer's disease, considered by many authors as accelerated brain aging (Coleman and Flood, 1987; Dr. D.F. Swaab, personal communication), the level of DNA breaks and alkali-labile sites was determined in cerebral cortex samples from Alzheimer's disease patients and controls.

This chapter deals with the present knowledge on the occurrence of "spontaneous" DNA damage in aging organisms, its potential sources, the influence of preventive and processive cellular defense mechanisms and its consequences in terms of DNA sequence changes, DNA conformational and configurational changes and changes in gene expression (Fig. 1.1).

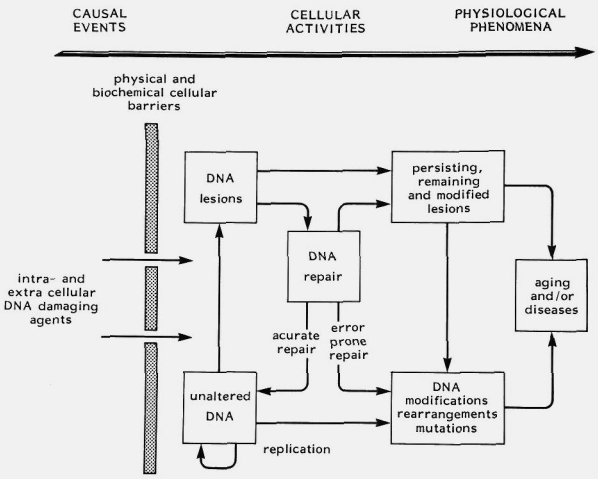

Figure 1.1: Possible effects of DNA damage and repair on cellular processes and aging.

1.2 Potential sources of DNA damage

1.2.1 Endogenous sources

Table 1.1 lists the various potential endogenous sources of DNA damaging agents and the effects these may theoretically have in terms of the frequency of induction of various types of DNA lesions in cells in vivo. It should be realized that the data provided in Table 1.1 are estimates, mostly based on experiments with isolated DNA; quantitative data on the spontaneous induction of endogenous DNA damage in cells and tissues of higher animals are still lacking, with the exception of recent results reported on the presence of 8-hydroxydeoxyguanine in rat liver DNA (Richter et al., 1988, see below).

Body heat. Perhaps the most ubiquitous natural cause of DNA damage is heat. Due to the thermodynamic instability of DNA, certain alterations in its structure can already occur at the normal human body temperature of 37°C. For instance, the N-glycosylic bond between the purine or pyrimidine base and the deoxyribose is relatively labile and can easily be broken at elevated temperature (37°C), resulting in apurinic or apyrimidinic sites (AP-sites). From experiments in which the release of labeled bases was measured in double stranded Bacillus subtilis DNA, incubated at different temperatures, Lindahl and Nyberg (1972) estimated that at the normal body temperature of 37°C about 10,000 depurinations are induced per mammalian cell per day. In isolated DNA at 37°C, such AP-sites are spontaneously converted into single-strand breaks (SSB) in about 100 h (Lindahl and Andersson, 1972). However, based on measurements from Crine and Verly (1976) of the spontaneous degradation of isolated T7 phage DNA in vitro (at 37°C) by using alkaline sucrose gradient centrifugation, Saul and Ames (1985) calculated that about 20,000-40,000 breaks per cell are induced per day. According to Crine and Verly (1976) most of the breaks found were generated by the spontaneous destruction of deoxyribose moieties or direct hydrolysis of phosphodiester bonds and not by depurination.

Table 1.1 ENDOGENOUS SOURCES OF DNA DAMAGE

Source	type of damage detected	Estimated amount of damage induced (per cell per day)[a]	Reference
Body heat (37°C)	SSB	20,000-40,000[b]	1
	apurinic sites	10,000[b]	2
	apyrimidinic sites	500[b]	3
	deamination	100-300[b]	4
Free radicals	SSB	?	
	DSB	?	
	apurinic sites	?	
	cross-links	?	
	thymine glycol	270[c]	5
	thymidine glycol	70[c]	5
	5-hydroxymethyluracil	620[c]	6
	8-hydroxydeoxyguanine	750[d]	7
Other sources	glucose adducts	3[b]	8
	N7-methylguanine	4,000[b]	9
	N3-methyladenine	600[b]	9
	O^6-methylguanine	10-30[b]	9
	cross-links	?	

[a] Calculated on the basis of 1.2 10^{10} nucleotides per mammalian cell.
[b] Based on experiments with isolated DNA.
[c] Based on data obtained from urine of humans.
[d] Based on the level found in livers of 6-month old rats, assuming a constant induction.

1) Crine and Verly, 1976; Saul and Ames, 1985; 2) Lindahl and Nyberg, 1972; 3) Lindahl and Karlström, 1973; 4) Lindahl and Nyberg, 1974; 5) Cathcart et al., 1984; 6) Saul et al., 1984; 7) Richter et al., 1988; 8) Cerami, 1986; 9) Rydberg and Lindahl, 1982.

At present no information is available as to the actual formation of SSB and AP-sites in tissues of higher animals in vivo. Since DNA in vivo is tightly packed together with proteins, the induction of damage might be quite different from the situation in vitro. Results from Bailly and Verly (1988) indicate, for example, that the presence of basic proteins such as spermidine and histone H1 promotes the conversion of AP-sites into SSB.

Other forms of heat-induced DNA alterations found in isolated DNA, include the deamination of cytosine to uracil and, to a smaller extent, the deamination of adenine and guanine to hypoxanthine and xanthine (Lindahl and Nyberg, 1974; Karran and Lindahl, 1980). In the cell, uracil, hypoxanthine and xanthine can be removed from the DNA enzymatically by specific glycosylases, resulting in AP-sites. Less frequently occurring heat-induced DNA modifications are caused by hydrolytic reactions such as the opening of the imidazole ring of adenine, hydration of pyrimidines and conversion of deoxyguanosine to deoxyneoguanosine (Lindahl, 1977).

Free radicals. A second mechanism acting as an endogenous source of DNA damage is the generation of free radicals during several metabolic processes in the cell. Important classes of free radicals are the active oxygen species superoxide (O_2^-), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^\cdot$). These radicals are generated during the sequential reduction of oxygen to water (Fig. 1.2; for a recent review, see Joenje, 1989). Also, during the oxidation of small molecules,

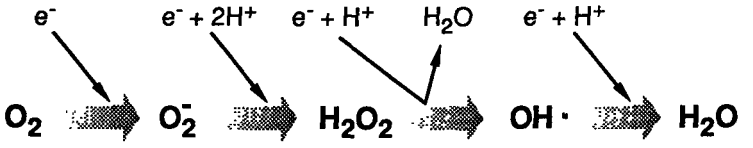

Figure 1.2: Generation of free radicals during the univalent reduction of O_2 to H_20.

such as thiols, hydroquinones and flavins, O_2^- radicals are formed (Freeman and Crapo, 1982). In addition, a number of enzyme systems, e.g. xanthine oxidase and aldehyde oxidase, generate free radicals as byproducts, while large amounts

of $O_2^{\cdot}$ radicals are liberated during active phagocytosis by polymorphonuclear leukocytes or granulocytes (Fridovich, 1978; Freeman and Crapo, 1982; Naqui et al., 1986).

Another important class of free radicals are lipid radicals. The reaction of free radicals with membranes can initiate a radical chain reaction leading to the oxidation of unsaturated lipids present in membranes and to the formation of lipid radicals, such as lipid-peroxy radicals, fatty acid hydroperoxides and cholesterol epoxides (Vaca et al., 1988).

The various types of radicals formed in the body can react with many cellular components, including DNA. Much knowledge concerning radical reactions with DNA comes from studies with ionizing radiation. The transfer of radiation energy (e.g. that of gamma-rays, X-rays and ultraviolet light) to cellular compounds, such as water, results in the formation of free radicals. Indeed, $OH^{\cdot}$ radicals are responsible for 70-80% of the SSB induced by gamma-rays (Roots and Okada, 1975; Repine et al., 1981). In DNA of cultured cells or in isolated DNA, gamma-ray-induced radicals give rise to a variety of oxidative damages, including SSB, double strand breaks (DSB), AP-sites and cross-links, but also to several modified bases such as thymine glycol and 5-hydroxymethyluracil, two forms of oxidized thymine (Cadet and Berger, 1985; Téoule, 1987).

Recent data indicate that small base damages are indeed formed in cells of higher animals in vivo. The modified bases mentioned above, and also thymidine glycol, have been found in the urine of rat and man, which is an indication for the continuous induction and removal of these DNA oxidation products in mammalian tissues (Cathcart et al., 1984; Saul et al., 1987). Interestingly, the amount of both thymine glycol and thymidine glycol per kg bodyweight found in the urine of short living animals (rats and mice) is higher than that in the urine of long living animals (monkey and man), suggesting a higher induction of oxidative DNA damage in the short living species (Adelman et al., 1988). This is in accordance with the idea that short living animals have a higher rate of oxygen metabolism per mass of bodyweight than humans (Cutler, 1986), which

may lead to a higher concentration of active oxygen species and an increased formation of oxidative damage. According to calculations made by Saul et al. (1987), about 1,000 of these DNA oxidation products (thymine glycols, thymidine glycols and 5-hydroxymethyluracil) are induced in each human cell per day (Table 1.1), whereas about 15,000 of such lesions are generated per day in the genome of a rat cell.

More direct evidence for the presence of oxidative DNA damage in cells in vivo, is the high level of 8-hydroxydeoxyguanine (140,000 molecules per cell) found in liver DNA from 6-month old rats (Richter et al., 1988). Interestingly, the level of this base damage found was about 16 times higher in mitochondrial DNA than in nuclear DNA (Richter et al., 1988), which is not unexpected since mitochondria are a major source of oxygen radicals.

Additionally, the presence of repair enzymes for specific base damages may in itself be considered as evidence for the frequent natural occurrence of the corresponding lesions in vivo. Indeed, DNA glycosylases, specific for 5-hydroxymethyluracil (Hollstein et al., 1984, Boorstein et al., 1987) or for thymine glycol (Higgins et al., 1987) have been found in mammalian cells.

Other sources. Other reactive species besides oxygen can be the cause of spontaneous DNA damage. Data from Bucala et al. (1984; 1985) indicate that the non-enzymatic reaction of glucose and other reducing sugars with the amino groups of DNA bases can lead to the formation of DNA adducts. Furthermore, the reaction of glucose 6-phosphate with the amino group of lysine leads to the formation of reactive intermediates capable of interacting with DNA (Lee and Cerami, 1987). The latter process might be the underlying cause of the covalent attachment of proteins to DNA (DNA-protein cross-links), the occurrence of which has been observed in various cells during aging (see section 1.5.1 and Bojanovic et al., 1970).

Another spontaneously occurring reaction is the alkylation of DNA by S-adenosyl-L-methionine (SAM), the normal intracellular methylgroup donor (Barrows and Magee, 1982; Rydberg and Lindahl, 1982). This reaction can lead to the

formation of N7-methylguanine, N3-methyladenine and small amounts of O^6-methylguanine (Table 1.1). The methylation of guanine and adenine bases causes a further destabilization of the N-glycosylic bond, resulting in an increased spontaneous cleavage and the formation of AP-sites (Lindahl and Nyberg, 1972).

1.2.2 Exogenous sources

Although endogenous sources of DNA damage now appear to be the most important (Hart et al., 1979; Ames, 1983; Gensler et al., 1987), various exogenous sources cannot be excluded (Table 1.2). In the latter case, the extent of exposure of human individuals is of course much more determined by lifestyle, occupation and place of residence than with the endogenous sources.

The human diet contains a great variety of natural mutagens and carcinogens, such as polycyclic aromatic hydrocarbons (PAH), aflatoxin B1 and nitrosamines (for a review, see Ames, 1983). These agents can react with DNA, thereby forming several types of damages including SSB, DSB and bulky adducts. Also cigarette smoke contains various carcinogenic compounds, including benzo(a)pyrene (BaP). DNA adducts induced by BaP, have been detected - by using the ^{32}P-postlabeling assay - in human placental tissue (Everson et al., 1986; Randerath et al., 1986a), bronchus samples (Randerath et al., 1986a) and lung tissue (Phillips et al., 1988a) of heavy smokers, but not or only at low levels in white blood cells (Perera et al., 1987) of such individuals. By using monoclonal antibodies against the reaction product of BaP diol-epoxide with guanine, DNA adducts were detected in bronchial cells of heavy smokers (Baan et al., 1988).

Another exogenous source of DNA damage is sunlight. The extent of exposure to sunlight is determined by both lifestyle and place of residence. The presently rather common recreational forms of sun exposure induce high levels of pyrimidine dimers, the main DNA lesion induced by the UV component of sun light, in the DNA of skin cells (Setlow, 1982; Niggli and Röthlisberger, 1988).

Table 1.2 EXOGENOUS SOURCES OF DNA DAMAGE

Source		Type of damage	Approximate number of adducts induced per cell[a]	Ref.
Life style	Sun bathing	Pyr	80,000[b]	1
		Pyr	60,000[b]	2
	Smoking	PAH	200[c]	3
		PAH	400-700[c]	4
		PAH	100-2,000[c]	5
Occupation	Coke oven workers	BPDE	7,000-70,000[d]	6
		BPDE	400 - 4,000[d]	7
	Foundry workers	BPDE	300-2,000[d]	8
		BPDE	1,000-6,500[d]	9
		PAH	10-1,300[d]	10
	Roofers	BPDE	200-10,000[d]	8
Place of residence	background radiation	SSB	2[e]	11

a Calculated on the basis of 1.2×10^{10} nucleotides per mammalian cell.
b Number of pyrimidine dimers induced during 1 h of sun exposure.
c Level of DNA adducts found in individuals who smoke about 20 cigarettes per day.
d Level of DNA adducts found in individuals working for several years in these facturies.
e Calculated number of SSB induced per year based on the estimated level of background radiation of 0.2 rem/year (Hennen, 1987; UNSCEAR, 1988) and an induction of 1,000 SSB per cell per Gy (Van der Schans et al., 1982)

Abbreviations: BPDE = Benzo(a)pyrene diol epoxide; Pyr = pyrimidine dimer; PAH = polycyclic aromatic hydrocarbon; SSB = single-strand break.

1) Setlow, 1982; 2) Niggli and Röthlisberger, 1988; 3) Everson et al., 1986; 4) Randerath et al., 1986a; 5) Phillips et al., 1988a; 6) Harris et al., 1985; 7) Haugan et al., 1986; 8) Shamsuddin et al., 1985; 9) Perera et al., 1988; 10) Phillips et al., 1988b; 11) Hennen, 1987.

Also occupational exposure to genotoxic agents can lead to the induction of DNA damage. High levels of BaP diol epoxide-DNA adducts were detected in lymphocytes of individuals known to be exposed to high levels of BaP, such as coke oven workers (Harris et al., 1985; Haugan et al., 1986), roofers and

foundry workers (Shamsuddin et al., 1985; Perera et al., 1988; Phillips et al., 1988b). Generally, the level of adducts in these individuals correlated with the exposure level, but the interindividual variation was large (Perera et al., 1988).

A further external source of DNA damage is formed by the natural background level of ionizing radiation, which is about 0.2 rem/year (2×10^{-3} Gy/year) (Hennen, 1987; UNSCEAR report, 1988). This irradiation induces about 5×10^{-3} SSB per cell per day, but there can be large variations in the extent of exposure. For instance, the level of radiation originating from radon (Rn-222), which is present in the earth but also in the building materials of our houses, can vary from 3×10^{-4} to 3×10^{-3} Gy/year (IARC Report, 1988; Nero, 1988).

Some chemicals and radiation can induce DNA damage indirectly, via the generation of free radicals (Ide et al., 1983; Lesko, 1984; Cerutti, 1985). As mentioned earlier (section 1.2.1) irradiation of cells with gamma-rays, x-rays or ultraviolet light can lead to the formation of radicals, which can induce DNA damage. However, during metabolic activation or detoxification of several carcinogens by mixed-function oxidases utilizing cytochrome P-450, various types of radicals are also generated, which can react with DNA (Miller, 1978; Ide et al., 1983; Lesko, 1984). In such cases it is possible that no carcinogen-DNA-adducts are detected, but only radical-induced DNA damage, such as SSB and base damage (see also Mullaart et al., 1989c, Chapter 5).

According to the "membrane-mediated DNA damage" theory of Cerutti et al. (1983), carcinogens can induce DNA damage via interaction with membranes. Binding of carcinogens to the membrane stimulates the arachidonic acid cascade, elicits an oxidative burst and disturbs the membrane structure. In this way active oxygen species as well as lipid peroxidation and aldehydic degradation products are generated and these bring the cell in a so called "pro-oxidant" state (Cerutti, 1985). These highly reactive secondary radicals can induce DNA damage, although the carcinogens themselves do not react with the DNA.

1.3 Molecular defense systems

1.3.1 Protection of DNA against damage induction

There are various systems to eliminate DNA-damaging agents before they can interact with the DNA. The enzyme superoxide dismutase (SOD) converts the O_2^- radical into hydrogen peroxide, which in turn, is converted into water by catalase and glutathione peroxidase. It should be noted that the levels of SOD, catalase and glutathione peroxidase can differ between different species (for review, see Cutler, 1984), which may partly explain the inter-species differences in levels of oxidative DNA damage that have been observed (Adelman et al., 1988). Besides these enzymatic defense systems, there are several substances generated in the cell or present in the food, that have the ability to scavenge free radicals. Examples of such protectors are selenium, vitamin E (α-tocopherol), vitamin C, glutathione and cysteine. Furthermore, mixed-function oxidases, present in the liver, play an important role in the detoxification of various carcinogens.

Increases in the capacity or efficiency of defense systems would be expected to lower the amount of DNA damage induced. Indeed, the relatively high level of DNA damage found in the liver of old mice could be lowered by feeding the animals with the antioxidants butylated hydroxyanisole and oltipraz for several weeks (Lawson and Stohs, 1985). Furthermore, the number of carcinogen-induced chromosomal breakage (probably induced via radicals) in blood lymphocytes is reduced by incubation of the cells with antioxidants such as selenium and vitamin E (Shamberger et al., 1973). Addition of glutathione esters or cysteamine to cultured Hela cells increased the intracellular glutathione level and made the cells more resistent against radiation damage (Vos and Roos-Verhey, 1988).

1.3.2 DNA repair

In spite of the elaborate "first line" of molecular defense systems, discussed above, it is generally believed that thousands of DNA lesions are induced in the DNA of each cell per day (Tice and Setlow, 1985; see also Tables 1.1 and 1.2). Fortunately, cells are equipped with a battery of repair systems to remove such damage (for an extensive review, see Friedberg, 1985). These repair systems can roughly be divided into 3 categories, i.e. direct repair, excision repair and post-replication repair. In direct repair, the lesion itself is removed without any further (transient) changes in the DNA structure. Direct repair includes the enzymatic photo-reactivation of UV-induced pyrimidine dimers (Eker, 1983) and the removal of O^6-methyl adducts by methyltransferase (Lindahl, 1982).

DNA-excision repair is brought about by a complex multi-enzyme system, the components of which are involved in the various steps in this repair process, viz. (1) recognition of the damage; (2) strand-nicking either by direct incision or via glycosylation; (3) removal by an exonuclease of a certain length of DNA of the chain containing the damage; (4) re-synthesis with the intact opposite strand as a template; and (5) ligation of the gap (for a review, see Haseltine, 1983). On the basis of the number of substituted nucleotides per repair patch in mammalian cells, this type of repair has been divided into short-patch (2-10 nucleotides) and long-patch (25-100 nucleotides) repair. Damage induced by UV, as well as that induced by "UV-like" chemicals (such as polycyclic aromatic hydrocarbons) is removed via long-patch repair, while damage induced by gamma-rays and "gamma-like" chemicals (such as alkylating agents) is repaired via short-patch repair (Regan and Setlow, 1974).

The third type of repair, post-replication repair, does not actually remove the damage but allows the replication system to pass or circumvent the damage. Post-replication repair systems (e.g. recombinational repair and "translesion synthesis") have been described for E. coli (Rupp and Howard-Flanders, 1968; Rupp et al., 1971), and have also been suggested for mammalian cells (Park

and Cleaver, 1979, Meneghini et al., 1981). It should be mentioned that translesion synthesis, the incorporation of nucleotides opposite damaged, i.e. non-informational, sites can easily lead to misincorporation (mutations).

The activity of DNA repair systems can vary greatly among different species and individuals. For instance, large species-specific differences were found in the amount of UV-induced excision repair, determined as "unscheduled DNA synthesis" (UDS), in fibroblasts isolated from 7 different species (Hart and Setlow, 1974). Interestingly, these differences correlated well with the maximum lifespan of the species and have therefore often been cited as an indication for the role of DNA repair in the aging process. However, data from Kato et al (1980) using 34 animals did not confirm this correlation. Results from Setlow (1983) indicate large inter-individual differences in the amount of UV-induced UDS between fibroblasts from various human individuals. Recent data from Boerrigter et al. (1989b) indicate large inter-individual differences in the rate of removal of ethyl nitrosourea-induced alkali-labile sites from human lymphocytes.

Besides these inter-species and inter-individual differences in repair activities, there are some hereditary disorders associated with a defect in certain DNA-repair activities. The most extensively studied DNA-repair defect in humans is that in xeroderma pigmentosum (XP) patients (Cleaver and Bootsma, 1975). Cells from XP patients are characterized by a defect in the repair of UV-induced pyrimidine dimers (and other DNA damages which are repaired via long-patch repair), leading to an increased risk of skin-tumor formation. Other human disorders that may be associated with a DNA-repair defect are ataxia telangiectasia, Fanconi's anemia and Bloom's syndrome (Cleaver, 1980). It has also been suggested that Alzheimer's disease (AD) is characterized by a defect in some aspects of DNA repair (Robbins, 1983; see also Mullaart et al., 1989e, Chapter 8).

It should be realized that DNA-repair systems are never 100 % efficient and that part of the damage may persist for a long time (see for example Chapters 2, 3 and 4). A high efficiency of DNA-repair systems will be needed to keep the level of persisting lesions low. However, as discussed by Kirkwood and Holliday

(1986) complete accuracy of DNA repair would eliminate the occurrence of evolutionary changes. Interesting within the framework of a possible role of DNA repair in aging, is the consideration that there may be a trade-off between the efforts required to maintain somatic integrity and function and the investment in reproduction. By mathematical modelling Kirkwood and Cremer (1982) showed that maximum fitness is achieved by balancing somatic maintenance against reproductive effort in such a way that the investment of resources and energy applied in maintenance and repair of the somatic cells is always less than what is required for indefinite longevity; it follows that aging is due to an accumulation of unrepaired somatic defects among which DNA changes may be prominent (Kirkwood, 1989).

Besides such general theoretical considerations about the advantages or disadvantages of perfect DNA-repair systems, it is not inconceivable that DNA-repair activities can be stimulated or repressed depending on the situation. Interestingly, Gupta and Sirover (1980) found a stimulation of DNA-repair systems in cultured human fibroblasts prior to DNA replication. After exposure to sodium bisulfite or to methyl methanesulfonate, the level of the enzyme uracil DNA glycosylase as well as the extent of UDS, appeared to change as a function of the cell cycle. A 5-fold enhancement in uracil DNA glycosylase levels and a 4- to 30-fold increase in the amount of UDS was found shortly before the initiation of DNA replication. The authors suggested that the function of such a stimulation at this point in the cell cycle might be the removal of pre-existing DNA lesions, which would otherwise interfere with DNA replication. This would imply that a cell can tolerate a certain level of DNA damage, and will remove this damage only then when it is really necessary. The preferential repair of transcribed DNA strands (Mellon et al., 1987) might be another example; first of all, those parts of the DNA which are essential from the point of view of survival are repaired.

1.4 Molecular endpoints of DNA damage

An important immediate "endpoint" of DNA damage is cell death, which may occur when lesions are induced in certain crucial genes or as a consequence of replicating a damaged template. Although cell death may contribute to aging and could be partly responsible for the decline in organ function with age, it is not likely to contribute heavily to many of the age-related diseases that bring life to an end. An exception could be neurodegenerative diseases, such as Alzheimer's disease, in which rapid degeneration and death of neurons take place. This is discussed in some more detail in Chapter 8.

However, it should be realized that the presence of DNA damage may have several deleterious effects on proper functioning of cells and tissues other than immediate cell death. DNA lesions can interfere with DNA and RNA synthesis in a way that leads to DNA-sequence changes and altered patterns of gene expression, respectively. The presence of DNA damage can also lead to changes in gene-expression by eliciting a so-called "stress response". Well-known examples of the latter are the SOS response in E. coli, the induction of highly active but not very accurate DNA repair mechanism (Radman, 1975), and the "heat-shock response" found in nearly all organisms (Ashburner and Bonner, 1979). The ultimate consequences of the responses of the cell to DNA damage, e.g. DNA sequence changes and alterations in gene expression, are discussed in some more detail below.

1.4.1 DNA sequence changes

DNA damage can inhibit normal DNA synthesis by blocking the progression of the replication fork or by inhibiting the initiation of the replication. Usually, directly after a genotoxic treatment, DNA replication is inhibited, but only temporarily; a few hours later, a restoration of the DNA synthesis can be observed. This restoration of DNA synthesis depends on the removal of DNA damage,

as is indicated by the lack of recovery of DNA synthesis in UV-irradiated xeroderma pigmentosum cells, which are unable to remove UV-induced DNA damage (Moustacchi et al., 1979). There are, however, indications that DNA replication can already start before all damage is removed. Systems for the replication of damaged DNA, so-called post-replication repair pathways, have been suggested to be operative in mammalian cells (Park and Cleaver, 1979; Meneghini et al., 1981). As discussed earlier (section 1.3.2), such post-replication systems may be error-prone; as a consequence, the replication of lesion-containing DNA can lead to point mutations and rearrangements. Indeed, replication in E. coli, in which SOS repair systems are activated (see section 1.4.2), of DNA containing O^6-methylguanine was shown to induce high numbers of predominantly GC=>AT mutations (Loechler et al., 1984), while unrepaired AP-sites lead to GC=>TA and AT=>TA mutations (Schaaper and Loeb, 1981; Loeb and Preston, 1986).

It has been suggested that the inhibition of DNA replication found after treatment of cells with DNA damaging agents is a protection mechanism, since it would give the cell more time to remove the lesions before DNA replication can fix the damage by inducing a mutation. Results from Konze-Thomas et al. (1982), indeed indicate that for confluent human fibroblasts the time between UV-irradiation and start of the replication, the latter induced by seeding at low density, determines the mutagenicity but not the cytotoxicity of the exposure. Therefore, it has been suggested by these authors that the mutation frequency is determined by the number of unrepaired lesions at the time of DNA replication.

The replication of DNA containing unrepaired double strand breaks (DSB) may lead to large DNA rearrangements (chromosomal aberrations) (Natarajan and Obe, 1978; Natarajan et al., 1980).

1.4.2 Alterations in gene-expression

Analogous to the inhibition of DNA synthesis, also RNA synthesis

(transcription) can be inhibited by DNA damage. Mayne and Lehmann (1982) demonstrated that the rate of RNA synthesis in normal human fibroblasts was depressed after irradiation with 4 J/m^2 UV-C, but recovered to control levels within 3-4 h. Since in normal human fibroblasts after this time period more than 50 % of the dimers are still present (Vijg et al., 1984), there seems to be no correlation between the recovery of RNA synthesis and the overall extent of excision repair. In addition, in UV-irradiated cultured fibroblasts from patients with Cockayne's syndrome, which have no detectable defect in overall excision-repair, RNA synthesis recovered only slowly (after 8 h) or not at all (Mayne and Lehmann, 1982). The preferential removal of DNA damage from actively transcribed parts of the genome (Bohr et al., 1985) might explain the discrepancy between the recovery of RNA synthesis and the incomplete removal of DNA damage. Indeed, Mullenders et al. (1988) found that cells from patients with Cockayne's syndrome have a diminished capacity to repair actively transcribed genes.

Besides by directly inhibiting gene transcription as a consequence of its location in a coding region, DNA damage can also influence gene expression by changing the configuration or conformation of the DNA. For instance, the methylation state of DNA, an important factor in the regulation of gene expression by modulating configuration of specific sequences (Cedar, 1988), can be influenced by DNA damage and repair. Normally, methyl transferases, operating on hemi-methylated DNA, restore the methylation pattern of the DNA within 2 h after each round of replication. However, in repair patches the restoration of the methylation pattern appeared to be much slower and incomplete; in confluent human fibroblasts, remethylation was prolonged over more than 3 days (Kastan et al., 1982). Furthermore, Kastan et al. (1982) found that UV-irradiation leads to a loss of methylation during subsequent rounds of replication. So, when the DNA replication starts before complete restoration of the normal methylation pattern, there is a definitive heritable loss of methylated sites.

DNA damage can also influence gene expression by interfering with the

protein-DNA interactions necessary for transcription; certain regulatory regions such as enhancers and promoters can be blocked. For example, results from Brown and Cerutti (1987) indicate that the inactivation of transfected SV-40 DNA is determined by the presence of damage in the regulatory region that governs viral gene expression.

It should be realized that lesions in the DNA may frequently result in some conformational change, which may have effects not only locally but also at some distance. According to Müller et al. (1988) transcription can be stimulated by the interaction of a remote enhancer with the promotor through the formation of a DNA loop via DNA-binding proteins. It is not inconceivable that the DNA conformation is an important factor in determining whether or not such a loop is formed. Due to the presence of DNA lesions, alterations in the torsional stress may occur and this can decrease the efficiency of DNA loop formation. In general this means that, in addition to completely abolishing gene expression by, for example, its presence in coding sequences, DNA damage can readily induce alterations in patterns of gene expression, which may ultimately lead to a loss of gene control.

The presence of DNA damage can also change patterns of gene-expression by the induction of a so-called "stress-response" in the cell, during which several proteins are induced or activated. The best known example of such a stress response is the SOS response in E. coli, which leads to an activation of excision and postreplication repair, an enhanced mutagenesis and the shut-off of cellular respiration (Radman, 1975). However, until now no data are available about the presence of such SOS systems in mammalian cells. A second well-known stress response is the heat-shock response, which involves the induction of several proteins as a reaction to exposure to elevated temperature; this phenomenon has been found in nearly all organisms (Ashburner and Bonner, 1979). There is some evidence that in Saccharomyces cerevisiae (McClanahan and McEntee, 1986) and perhaps also in mammalian cells (Fornace et al., 1989), some of these heat-shock proteins are induced by DNA-damaging agents. Recently, data became

available on the induction of a large variety of "stress proteins" in mammalian cells by DNA-damaging agents. Results from Fornace et al. (1988) indicate the induction of several transcripts in cultured Chinese hamster cells following UV-irradiation or treatment with H_2O_2, methyl methanesulfonate or N-acetoxy-2-acetylaminofluorene. One of these transcripts is similar to a heat-shock protein, while another shows homology with a helix-destabilizing protein (Fornace et al., 1988). In addition, Keyse and Tyrrell (1987) observed the induction of several "stress proteins" in cultured human skin fibroblasts after treatment with H_2O_2 or irradiation with near-UV (320-380 nm). They suggest that this induction may be related to the cellular oxidant stress situation (high levels of oxygen radicals) induced by these agents.

Another example of stress response in mammalian cells is the activation of the enzyme ADP-ribosyl transferase (ADPRT). The activity of this enzyme, which catalyzes the transfer of the ADP-ribose moiety of NAD^+ to various nuclear proteins (Fig. 1.3), is greatly stimulated by the presence of SSB (Ueda, 1985).

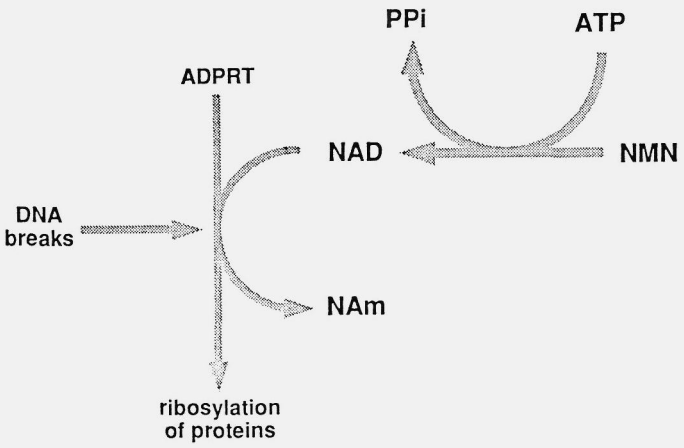

Figure 1.3: Cellular effects of the activation of ADPRT by DNA damage. ADPRT = ADP-ribosyl transferase; ATP = adenosine triphosphate; NAD = nicotinamide dinucleotide; NAm = nicotinamide; NMN = nicotinamide mononucleotide; PPi = diphosphate.

During the successive ribosylation of several proteins, large amounts of NAD^+ and ATP are consumed (Fig.1.3), which eventually leads to cell death. It has been postulated that this "suicidal" system of eliminating cells that contain a large amount of DNA damage contributes to the survival of the organism, since repair of so much damage could easily lead to the induction of mutations (Carson et al., 1987).

1.5 Accumulation of DNA changes during aging: What's the evidence?

From the data discussed earlier (section 1.2) it can be concluded that the cells of lower and higher organisms are continuously threatened by DNA-damaging agents. As described in section 1.3, the cell is equipped with several molecular defense systems which can either prevent the induction of damage or remove the damage induced. Apparently, these defense systems are not always perfect and this can easily lead to a gradual accumulation of DNA damage or secondary DNA changes with age. Furthermore, an age-related decline in the efficiency of these defense systems can accelerate the rate of such an accumulation during aging. An age-related decline of antioxidant systems has been reported (for reviews, see Sohal and Allen, 1985; Benzi et al., 1989), although there are exceptions. For instance, Scarpa et al. (1987) found an age-related increase in both SOD and glutathione peroxidase levels in rat brain. An impressive number of experiments have been devoted to examining the possible existence of a decline in DNA-repair activities with age (for a recent review, see Tice and Setlow, 1985; Hanawalt, 1987). The general conclusion from these data, mostly obtained with cultured cells, is that there is no age-related decline in the efficiency of DNA-repair systems (see also section 1.7). Also our own data (Chapter 3 and 4) indicate a lack of subtantial age-related changes in DNA repair activities.

Nevertheless, the sole fact that molecular defense systems are not perfect, necessitates direct studies on the possibility that DNA damage and other secondary changes accumulate with age. In the following sections an overview will be given

of the data available at present on the accumulation of several forms of DNA damage, i.e. SSB, base damage and cross-links with age. In addition, some direct evidence for the occurrence of changes in sequence organization or gene-expression in higher organisms as a function of their age will be briefly discussed.

1.5.1 DNA damage

DNA breaks/alkali-labile sites. Various attempts have been made to demonstrate an age-related increase in the percentage single-strand breaks (SSB) or damage such as AP-sites, that are converted into DNA breaks during treatment with alkali (alkali-labile sites) (Table 1.3). From these studies it appears that the occurrence of an age-related accumulation of DNA damage depends on the organ or tissue studied, but also on the assay system used. In this respect it should be realized that the different aspecific assay systems presently in use detect different forms of DNA damage (for a review, see Ahnström, 1988). By using alkaline-sucrose gradient centrifugation, alkaline elution and alkaline unwinding, SSB, DSB and alkali-labile sites can be detected. The S_1-nuclease assay only detects SSB, DSB or stretches of single-stranded DNA. By the nucleoid sedimentation assay, in which the sedimentation rate of nucleoids through a neutral sucrose gradient is determined, only SSB and DSB can be detected. This latter assay, however, can easily be influenced by other alterations in the DNA, such as conformational changes (Boerrigter et al., 1989; Jostes et al., 1989). These differences should be kept in mind when interpreting the results with these (aspecific) assay systems.

In the liver an age-related increase in the level of SSB and/or alkali-labile sites was found when measured by alkaline sucrose gradient centrifugation (Ono et al, 1976; Su et al., 1984) or alkaline elution (Lawson and Stohs, 1985; Mullaart et al., 1988b, Chapter 6). However, Mullaart et al. (1988b, Chapter 6) demonstrated

Table 1.3 AGE-RELATED CHANGES IN THE PERCENTAGE OF SINGLE-STRAND DNA BREAKS AND/OR ALKALI-LABILE SITES.

Organ/cell type	Species	Assay	Result	Reference
Brain	Mouse	DPTA	Increase	1
	Dog	AZC	Increase	2
	Mouse	AS	No increase	3
	Mouse	AS/S_1N	Increase	4
	Mouse	IF	Increase	5
	Mouse	S_1N	Increase	6
	Mouse	AS	No increase	7
	Rat	AE	No increase	8
	Rat	NS	Increase	9
Liver	Mouse	DPTA	Increase	1
	Mouse	HM	Increase	10
	Mouse	S_1N	Increase	11
	Mouse	AS	Increase	3
	Mouse	S_1N/CG/HM	No increase	12
	Mouse	S_1N	No increase	13
	Mouse	IF	Increase	5
	Mouse	S_1N	No increase	6
	Mouse	AS	Increase	7
	Mouse	AE	Increase	14
	Rat[a]	AE	Increase	15
	Rat[b]	AE	No increase	15
	Rat	NS	Increase	9
Kidney	Mouse	S_1N	No increase	6
	Mouse	AS	Increase	7
Muscle	Rat	AS	Increase	16
Intestine	Mouse	IF	No increase	5
	Rat	NS	No increase	9
Spleen	Mouse	AS	No increase	3
	Mouse	S_1N	No increase	6
Thymus	Mouse	AS	No increase	3
Heart	Mouse	DPTA	Increase	1
	Mouse	CS	Increase	17
	Mouse	S_1N	No increase	6
Lympho-cytes	Human	NS	Increase	18
	Human	AE	No increase	19

[a] parenchymal rat liver cells.
[b] non-parenchymal rat liver cells
Abbreviations: AE = alkaline elution; AS = alkaline sucrose gradient sedimentation; AZC = alkaline zonal centrifugation; CG = cesium chloride gradient sedimentation; DPTA = DNA polymerase template activity; IF = immunofluorescence with anti-cytidine antibody; HM = hydrodynamic measurement; NS = nucleoid sedimentation; S_1N = S_1 nuclease sensitivity.

1) Price et al., 1971; 2) Wheeler and Lett, 1974; 3) Ono et al., 1976; 4) Chetsanga et al., 1977; 5) Nakanishi et al., 1979; 6) Mori and Goto, 1982; 7) Su et al., 1984; 8) Mullaart et al., 1989d; 9) Hartnell et al., 1989; 10) Massie et al., 1972a; 11) Chetsanga et al., 1975; 12) Dean and Cutler, 1978; 13) Finch, 1979; 14) Lawson and Stohs, 1985; 15) Mullaart et al., 1988b; 16) Karran and Omerrod, 1973; 17) Chetsanga et al., 1976; 18) Hartwig and Körner, 1987; 19) Boerrigter et al., 1989b.

that such an age-related increase in DNA damage in rat liver only takes place in post-mitotic parenchymal cells and not in the non-parenchymal liver cells, which still have proliferative activity.

Chetsanga et al. (1975), found a large age-related increase (5 fold) in the fraction of DNA sensitive to S_1-nuclease in mouse liver, indicating an increase in the percentage of single-stranded DNA. However, using the same assay but other mouse strains, no such an age-related difference in the level of single-stranded DNA was observed in liver (Dean and Cutler, 1978; Finch, 1979; Mori and Goto, 1982).

No age-related difference in the level of SSB and alkali-labile sites could be detected in mouse brain by using alkaline sucrose gradients (Ono et al., 1976; Su et al., 1984) or in rat brain with the alkaline elution (Mullaart et al., 1989d, Chapter 7). This is in agreement with data from Walker and Bachelard (1988), which indicate the absence of an age-related difference in the level of SSB detected by nucleoid sedimentation in rat brain nuclei. However, using the S_1-nuclease assay Chetsanga et al. (1977) demonstrated an age-related difference in the level of single-stranded DNA in rat brain. In addition, the extent of unwinding of DNA in alkali, a measure for the amount of SSB and alkali-labile sites, was found to increase with age both in rat brain and in liver cells, but not in intestine cells (Hartnell et al., 1989).

Taken together, these findings suggest an age-related increase in DNA breaks and/or alkali-labile sites in rodent liver; in brain the majority of the data suggest an absence of an age-related change. For other organs and tissues not enough data are available to draw any conclusion about the occurrence of an age-related accumulation of DNA breaks or alkali-labile sites.

Base damage. Only limited data are available on the levels of specific base damage in relation to *in vivo* aging. Randerath et al. (1986b), using the ^{32}P-postlabeling assay, found an age-related accumulation of several unknown DNA adducts (I-spots) in rat liver, kidney, heart and lung of 10-month old rats as compared to 1-month old animals. The highest level of adducts (1,000 adducts/cell)

was found in liver and kidney DNA (Randerath et al., 1986b; Randerath et al., 1988, Randerath et al., 1989), while low levels of adducts were present in brain DNA (K. Randerath, personal communication). Unfortunately, no information is available about the level of these I-spots in rats older than 10 months. Using the same assay, Gaubatz (1989) found about 15,000 unknown aromatic adducts per cell in heart tissue of 39-month old mice compared to about 1,200 adducts in 2-month old animals.

Until now the identity of the I-spots is not known, although they do not appear to comigrate with any known carcinogen-induced DNA-adduct (Randerath et al., 1988). It is nevertheless still possible that they are induced via metabolically activated exogenous carcinogens or stem from metabolically formed, possibly hormone-associated, endogenous electrophiles (Randerath et al., 1988).

Cross-links. Some data are available on the age-dependency of the level of interstrand DNA-DNA cross-links and DNA-protein cross-links (for a review, see Tice and Setlow, 1985). With highly purified liver DNA from rats or mice, no age-related difference in thermal stability or template activity, both measures for the amount of cross-linking, was found (Russell et al., 1970; Kurtz et al., 1974). These findings suggest the absence of an age-related increase in DNA-DNA cross-linking. However, by using crude preparations from rat and mouse liver Russell et al. (1970) found an age-related increase in thermal stability and a decrease in template activity. This might indicate that there is an age-related increase in the level of DNA-protein cross-linking. In addition, using mouse liver cells Bojanovic et al. (1970) and Acharya (1972) found an age-related increase in the number of protein-DNA cross-links, measured by the salt extractability of proteins from chromatin preparations.

Sharma and Yamamoto (1980) recorded an age-dependent increase in the amount of a highly fluorescent modified base product in mouse liver DNA after hydrolysis. This modified base was similar to that induced by irradiation of DNA with gamma-rays in the presence of oxygen and was identified as a DNA-DNA cross-link product (Yamamoto et al., 1988).

1.5.2 DNA sequence changes

As discussed earlier (see section 1.4.1), the disturbance of DNA replication by the presence of DNA damage can easily lead to changes in the DNA sequence organization, i.e. mutations and rearrangements. The evidence that such molecular endpoints of DNA damage increase with age will be briefly discussed (for an extensive review, see Slagboom and Vijg, 1989).

Mutations occurring in human cells in vivo can be monitored by using the hypoxanthine phosphoribosyl transferase (hprt) assay (Albertini et al, 1982). This method is based on the enumeration and clonal expansion of cells mutated at the hprt locus, which allows growth in culture media containing 6-thioguanine, a substance that is toxic for cells with an intact hprt gene. By using this assay an age-related increase in the mutant frequency at the hprt locus was observed in human lymphocytes (Evans and Vijayalaxmi, 1981; Morley et al., 1982; Trainor et al., 1984; Vijayalaxmi and Evans, 1984; Albertini et al., 1988, Tates et al., 1989). No age-related increase in mutant frequency was found in mouse kidney and muscle cells (Horn et al., 1984).

The age-dependency of the number of chromosomal aberrations, i.e. chromosome breaks, chromatid-type lesions, etc. has been studied in actively dividing bone marrow cells and kidney cells, and in lymphocytes and liver hepatocytes, which can be stimulated to proliferate by means of phytohemagglutinin or partial hepatectomy, respectively. All reports on regenerating liver parenchymal cells of several species (mice, guinea pigs and Chinese hamsters) revealed a 2- to 5-fold age-related increase in the number of spontaneous chromosomal aberrations (Crowley and Curtis, 1963; Curtis and Miller, 1971; Brooks et al., 1973). In addition, a 6-fold increase in the frequency of chromosomal aberrations during aging was observed in mouse kidney cells (Martin et al., 1985). Recent studies from Marlhens et al. (1986) and Prieur et al. (1988) involving more than 1,000 metaphases from lymphocytes of 4 young and 2 old human individuals indicate an age-related increase in the number of chromosome breaks and chromatid-

type lesions.

An increased level of spontaneously-occurring sister chromatid exchanges (SCE), in human lymphocytes from old donors was found by several investigators (De Arce, 1981; Schmidt and Sanger, 1981; Waksvik et al., 1981; Dutkowski et al., 1985). However, neither Morgan and Crossen (1977) nor Schneider and Monticone (1978) found such an age-related increase in the frequency of SCE. This discrepancy might be explained by the fact that the level of SCE detected can be influenced by the culture conditions. The only study performed until now on cells in vivo , i.e. on bone marrow cells from both rat and mouse, did not reveal age-related variations in the number of SCE (Schneider et al., 1982).

1.5.3 Changes in gene-expression

Although RNA synthesis in general declines during aging (for a review, see Richardson et al., 1983), the presently available data on levels of specific mRNAs as a function of age are less clear; levels of some mRNAs decline with age, others stay the same, while in certain specific cases increased mRNA levels have been found (for a recent review, see Slagboom and Vijg, 1989). In this respect it should be mentioned that changes in specific mRNA levels could be adaptive, for example, in response to tissue damage, hormonal changes, etc.

In addition to the age-related changes in specific mRNA levels there is evidence for a general loss of transcriptional control during aging. For instance, such a relaxation of gene control has been hypothesized by Cutler (1985) in his dysdifferentiation theory and more recently by Vijg (1987) in the context of a general loss of DNA integrity during aging. Evidence for the loss of gene control during aging was provided by Ono and Cutler (1978), who demonstrated the appearance of globin mRNAs in brain and liver of old mice, which are not present in young animals. More recently, Slagboom et al. (1989) found an age-related increase in the inter-individual variability of tyrosine aminotransferase and ornithine transcarbamoylase expression in rat liver, which might be an

indication for a loss of transcriptional control during aging. As described in section 1.4.2, such a loss of transcriptional control could be due to the (transient) presence of DNA lesions in a variety of ways. One possibility is a gradual loss of DNA methylation, which has indeed been observed in various tissues of aging mice (Wilson et al., 1987) and in lymphocytes of old human donors (Drinkwater et al., 1989). However, DNA methylation patterns of specific genes appears to stay unaltered during aging (for a review, see Slagboom and Vijg, 1989). Some evidence has also been obtained for DNA conformational changes occurring with age in rat brain (Chaturvedi and Kanungo, 1983). The induction of stress proteins during aging, for example, as a consequence of DNA damage, has thusfar not been reported.

1.6 Recapitulation and Discussion

From the overview provided above a number of general conclusions can be drawn. First, a large variety of DNA lesions is likely to be induced "spontaneously" in the DNA of higher organisms at a rate that may differ among tissues, individuals and species; defects in the processing of DNA damage may be responsible for some specific human diseases. Second, the rate of damage induction as well as the persistency of the lesions depends on the activity, efficiency and reliability of a wide variety of molecular defense systems. A certain degree of imperfection seems to be a general characteristic of most of these defense systems. Third, even when they are quickly removed, DNA lesions can lead to secondary changes in the DNA, such as DNA-sequence changes and changes in gene expression. Finally, but most notably, in spite of a number of discrepancies and conflicting results, evidence has been obtained for the induction of DNA alterations with age at all levels, e.g. DNA damage, DNA sequence organization and gene-expression.

Nevertheless, uncertainties still exist with respect to the actual occurrence and the rate of some of these age-related changes. In many cases, this appears

to be due entirely to a lack of sophisticated techniques in studying low-frequency alterations in biomolecules in vivo in relation to aging. It is this aspect that needs some more discussion. Techniques to monitor, for instance, the level of "spontaneous" DNA damage, must be highly sensitive. From Table 1.1 it can be calculated that about 35,000-55,000 DNA lesions may be induced per cell per day (corresponding to about 10-15 lesions per 10^9 mw of DNA). However, most of these lesions, such as SSB, will be repaired rapidly. Based on data from experiments using cultured cells treated with various carcinogens, Lohman et al. (1985) estimated that the adduct level at which 37 % of the cells survive (on average one lethal lesion per cell is present), lies between 1 and 250 adducts per 10^9 mw DNA. It should be realized that cell division may force the cell to remove lesions that would otherwise interfere with DNA replication. Since in post-mitotic cells this immediate need to remove lesions is absent, such cells might tolerate and accumulate higher levels of DNA damage before they become lethal. However, for aging other endpoints than cell death can be much more important (section 1.4). In any case the need for highly sensitive and accurate assay systems for monitoring DNA lesions and secondary changes in vivo with age, is a compelling one.

It should also be realized that DNA damage is often induced during the isolation and analysis of the DNA. For instance, the number of DNA breaks per cell found in the liver of young (6-month old) mice, by using alkaline sucrose gradient centrifugation is already 12,000-50,000 (Ono et al., 1976; Su et al., 1984; Mullaart et al., 1989e; Chapter 8), while with the more sensitive alkaline elution assay only about 800 breaks per cell were found (Mullaart et al., 1988b, Chapter 6). This could indicate that most of the breaks found with the alkaline sucrose gradients are actually an artefact of the method, which makes it difficult to correctly interpret the results obtained.

Another problem, especially with techniques for monitoring DNA sequence changes, is their sole applicability to cultured cells. An exception is the hprt-assay commonly used for mutation analysis. However, this technique can only

be applied to cells that are able to divide in culture and is prone to artefacts (Featherstone et al., 1987). Only recently, methods have been developed to monitor mutation frequencies in every cell type in vivo (Lohman et al., 1987; Vijg and Uitterlinden, 1987; Gossen et al., 1989).

In general, it can be expected that on the short term, the application of new ultrasensitive assay systems will lead to more information concerning the age-related occurrence and accumulation of primary and secondary changes in various organs and tissues of higher organisms.

1.7 Objective and design of the experimental work

The studies described in the experimental part of this thesis were aimed at investigating the possibility that, in rats, the capacity to remove specific DNA lesions declines as a function of age. Furthermore, the possibility was investigated whether the background level of DNA breaks and alkali-labile sites in liver and brain would increase with age. Finally, the level of background DNA breaks and alkali-labile sites in brain samples of Alzheimer's disease (AD) patients was determined and compared to that of elderly non-AD patients.

First, the possibility was considered whether cells may alter their repair characteristics when transferred from the in vivo situation to a cell-culture system. For this purpose the modified UV-endonuclease assay (Vijg et al., 1986a), which makes it possible to monitor the induction and removal of UV-induced pyrimidine dimers in cells in vivo, was used with rat epidermal skin cells as experimental model. The results obtained indicate that epidermal skin cells undergo a strong reduction in their capacity to remove pyrimidine dimers when they are transferred into culture (Chapter 2).

To examine whether there is an age-related decline in the DNA-repair capacity, the removal of UV-induced pyrimidine dimers was studied in epidermal skin cells from young and old rats both in vitro and in vivo (Chapter 3), using the

modified UV-endonuclease assay. The results obtained indicate the absence of an age-related decline in the capacity to remove pyrimidine dimers both in vitro and in vivo.

In order to be able to extend our studies on possible age-related changes in DNA-repair activities of cells in vivo to other agents and tissues, the induction and removal of DNA damage induced by the model genotoxic agent 2-acetylaminofluorene (AAF) was studied in liver cells from young and old rats (Chapter 4). In these experiments the ^{32}P-postlabeling assay was used. This ultrasensitive assay, which is based on the chromatographical separation of ^{32}P-labeled modified bases, can detect 1 damaged base per 10^9 unmodified bases (3 adducts/10^{12} mw of DNA) (Gupta, 1985; Reddy and Randerath, 1986); it is particularly useful for the detection of aromatic-DNA adducts. Using this method only a slight (18%) age-related decline in the rate of disappearance of the AAF-induced DNA damage was observed (Chapter 4).

A complicating factor in testing the hypothesis that DNA damage accumulates with age is the lack of knowledge about the type of DNA lesions that may accumulate. From Table 1.1 it can be concluded that lesions induced by heat and free radicals, such as SSB and AP sites, are good candidates, since these occur at a high frequency. As demonstrated in Chapter 5, also environmental carcinogens such as benzo(a)pyrene induce SSB and/or alkali-labile sites, possibly via free radicals. Therefore, we monitored such forms of DNA damage, by using the alkaline elution assay, in rat liver and brain in relation to age. An important aspect of this assay is its high sensitivity (detection of 0.1 adducts/10^9 mw of DNA is possible) and its applicability to freshly isolated cells or nuclei (Stout and Becker, 1982). The results obtained by using this assay indicate an age-related increase in the level of SSB and/or alkali-labile sites in post-mitotic rat liver parenchymal cells but not in non-parenchymal liver cells, that still have proliferative capacity (Chapter 6). In addition, no age-related changes in the level of SSB and/or alkali-labile sites have been observed in rat brain cells (Chapter 7).

In the final part of the study the hypothesis was tested that Alzheimer's disease (AD) is correlated with an elevated level of DNA damage in the cerebral cortex (Chapter 8). It has been suggested that AD is actually an acceleration of the aging process normally occurring in the brain. According to the hypothesis of Robbins (1983), this might be caused by a DNA-repair defect leading to an increased rate of gamma-like DNA damage accumulation in AD brains. Therefore, the level of DNA damage was measured, by means of the alkaline elution assay, in cerebral cortex tissue samples from AD patients and controls of about the same age. The results obtained indicate an almost two fold higher level of breaks and/or alkali-labile sites in the cortex of AD patients as compared to the cortex of controls of about the same age.

CHAPTER 2

DIFFERENCES IN PYRIMIDINE DIMER REMOVAL BETWEEN RAT SKIN CELLS IN VITRO AND IN VIVO [1]

2.1 Introduction

The persistency of ultraviolet (UV)-induced pyrimidine dimers in cultured rodent cells is well documented (Lohman et al., 1976; Yagi, 1982; Ganesan et al., 1983; Takebe et al., 1983; Vijg et al., 1984). This persistency is not likely to be due to an inherent DNA repair defect of rodent cells, as can be deduced from data on the survival of such UV-irradiated cells, which is not lower than that of human cells under comparable conditions (Yagi, 1982; Ganesan et al., 1983). In addition, the amount of unscheduled DNA synthesis (UDS) performed by rodent cells over a given time period is too high to match the low number of dimers removed (Lohman et al., 1976; Yagi, 1982; Vijg et al., 1984). Interestingly, it has been shown by Peleg et al. (1977), and more recently by La Belle and Linn (1984), that early passage embryonic mouse cells remove pyrimidine dimers well. These findings suggest that in rodents the excision repair pathway(s), similar to those via which UV damage is repaired in human cells, can be activated but are not always fully utilized, possibly due to changes in the expression of specific DNA repair genes during embryonic development (Peleg et al., 1977).

Recently, we showed that UV-induced pyrimidine dimers in early passage fibroblasts from rat embryos were as persistent as those in cells from adult rats (Vijg et al., 1986a). In this regard it is conceivable that rat fibroblasts lose their capacity to remove dimers upon establishment into culture. In order to test this possibility it is necessary to measure the induction and removal

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[1] This work has been published previously by Mullaart et al., 1988a.

of pyrimidine dimers in cells in vivo, in comparison to the same cell type cultured in vitro. In principle, such a comparative study can be performed by using the UV-endonuclease enzymatic method (Paterson et al., 1973). However, a serious disadvantage of this analysis of the breaks induced by the enzyme, which involves centrifugation method is its dependence on radioactively labeled DNA for the analysis of the breaks induced by the enzyme, which involves centrifugation through alkaline sucrose gradients. This limits its application to growing cells cultured in vitro, the DNA of which can be labeled during S-phase with radioactive precursors. Modifications that circumvent the need of detection by radioactivity and therefore allow application of the alkaline sucrose gradient centrifugation technique to non-dividing cells or freshly isolated tissues, are based on the detection of DNA in the gradient fractions by fluorescent staining (Ono and Okado, 1973; Brash and Hart, 1983). Unfortunately, this modification makes the method rather time-consuming, because the DNA in each gradient fraction has to be precipitated, stained and spectrophotometrically quantified. An alternative is the detection of UV-endonuclease sensitive sites (ESS) by alkaline agarose gels (Sutherland and Shih, 1983).

Recently, a rapid and sensitive method for the determination of nonradioactively labeled alkaline sucrose gradient DNA profiles was developed (Vijg et al., 1986b). It is based on the fractionation of gradients in the wells of plastic microtiter plates and the subsequent covalent labeling of DNA adsorbed to the walls by reaction of the guanines with N-acetoxy-2-acetylaminofluorene (N-AcO-AAF). Then the treated DNA is quantified by means of an enzyme-linked immunosorbent assay (ELISA) (Engvall, 1980), with specific antibodies of high affinity towards dG-AAF. The method was found suitable for the accurate and sensitive detection of pyrimidine dimers induced in vitro as well as in vivo in rat skin cells by irradiation with UV. Further improvement was obtained when antibodies with a high affinity for DNA became available, which made the direct immunochemical detection

of DNA possible without the necessity of prelabeling.

The availability of the above described method allows an unbiased comparison of the repair of pyrimidine dimers in skin cells in vitro with that in vivo. Here we show that epidermal cells in vivo repair pyrimidine dimers rapidly, whereas in the same cell type in vitro these UV-induced lesions are as persistent as in cultured fibroblasts.

2.2 Material and Methods

2.2.1 Cell isolation and culture

Epidermal keratinocytes were isolated and cultured as described by Rheinwald and Green (1975), with some modifications. A skin biopsy was taken from the back of a narcotized rat (female WAG/Rij) and the epidermis was separated from the dermis by overnight floating (with the horny layer up) on 0.25% trypsin, 5 mM EDTA in PBS at 4°C. After this period the epidermis was removed and an epidermal cell suspension was obtained after thorough disaggregation by pipetting up and down. The cells were seeded on top of a feeder layer of lethally irradiated (30 Gy of ^{60}Co-gamma-rays) rat fibroblasts. The cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Gibco) supplemented with 10 % fetal calf serum, hydrocortisone (0.4 ng/ml) and cholera toxin (0.1 nM). Epidermal growth factor (10 ng/ml) was added after 2 days. The cells were used for repair experiments within 10 days after isolation.

Rat fibroblasts were obtained from the dermis of the same skin biopsies as described earlier (Vijg et al., 1984) and used between passage 5 and 10 as an exponentially dividing population (Vijg et al., 1984).

2.2.2 Determination of pyrimidine dimers after irradiation in vitro

The keratinocytes were labeled with ^{14}C-thymidine (1 μCi/ml, 56,5 mCi/mmol, Amersham) and the fibroblasts with ^{3}H-thymidine (1 μCi/ml, 25 Ci/mmol, Amersham). After 3 days of labeling and one day of incubation in non-radioactive medium, the medium was removed and the cells were washed with phospate buffered saline (PBS). The cells were irradiated on ice with UV-B (wavelenght range 290-320 nm), by using a Philips TL-20/12 fluorescent sunlamp at a fluence rate of 11 W/m^2, through a Schott 5-mm WG 305 filter (mimicking the horny layer). The fluence rate of the lamp was determined, before the filter, by means of a radiant flux meter (Hewlett Packard). During irradiation the cells were just covered with PBS containing 1 mg/ml glucose. After irradiation, the cells were incubated with fresh medium for 0, 3 and 24 h periods, washed with PBS and immediately frozen on dry ice. The feeder layer was removed from the keratinocyte culture by rinsing thoroughly with PBS containing 0.02% EDTA before freezing. The cells were lysed in 0.5 % (w/v) SDS, 150 mM NaCl, 10 mM EDTA, 20 mM Tris (pH 7.6) and 0.5 mg/ml proteinase K for 1 h at 37°C. After isolation by phenol extraction, DNAs from identically treated keratinocyte and fibroblast samples were mixed and the numbers of dimers were determined, after incubation of the DNA with a Micrococcus luteus UV-endonuclease, by alkaline sucrose gradient centrifugation as described earlier (Paterson et al., 1973; Wade and Lohman, 1980). In this way the number of dimers could be compared on the basis of the two distinguishable sedimentation profiles in the same gradient.

2.2.3 Determination of pyrimidine dimers after irradiation in vivo

For the determination of pyrimidine dimers in vivo a narcotized rat (female WAG/Rij) was shaved and irradiated with the Philips sunlamp for various periods of time (up to 10 min). Skin biopsies of 10 mm diameter were taken

and the epidermal cells were isolated as described above. To measure the removal of pyrimidine dimers from rat skin, biopsies were taken at 0, 3 and 24 h after UV-irradiation. During the repair period the rats were kept in the dark to exclude the possibility of photoreactivation repair.

The epidermal cells were lysed in 0.5 % (w/v) SDS, 150 mM NaCl, 10 mM EDTA, 20 mM Tris (pH 7.6) and 0.5 mg/ml proteinase K for 3 h at 55°C under continuous rocking. After isolation of the non-radioactively labeled DNA by means of phenol extraction, the number of ESS was determined essentially as described earlier (Vijg et al., 1986b), but with some modifications. After incubation with the M.luteus extract, the DNA was treated with proteinase K (1 mg/ml) for 1 h at 37°C and subsequently extracted once with 2 volumes of phenol, saturated with 0.2 M Tris-HCl, pH 7.6, followed by overnight dialysis against UV-endo buffer (Wade and Lohman, 1980) to remove the phenol. The number of ESS was determined by alkaline sucrose gradient centrifugation as described earlier (Vijg et al., 1986b).

2.2.4 Gradient fractionation and detection of DNA

The procedures for fractionation, liquid-scintillation counting and computer analysis of the DNA distributions obtained with radioactively labeled cells have been described (Wade and Lohman, 1980). Non-radioactive detection of DNA profiles was essentially as described earlier (Vijg et al., 1986b), but with some modifications. After centrifugation the sucrose gradients were fractionated into 96-well polystyrene microtiter plates (Greiner) at 8 drops (about 200 µl) per well and neutralized by adding 60 µl of neutralization buffer (1.3 M KH_2PO_4, 1.7 M Na_2HPO_4, pH 6.9). Directly thereafter, 100 µl was taken out of each well and spotted in a new microtiter plate which had previously been coated with poly-L-lysine (1 µg/ml PBS, overnight at 4°C). Subsequently the DNA was allowed to adsorb to the surface of the coated wells overnight at room temperature. The amount of DNA bound to the wells

was detected by means of an enzyme-linked immunosorbent assay (ELISA) (Engvall, 1980) involving a mouse monoclonal antibody against single-stranded DNA.

2.3 Results

When mammalian skin is irradiated with UV, the radiation will not penetrate deeply. In human skin, for example, only about 20-30 % of UV-B will reach the dermis; fibroblasts are thus not likely to be severely damaged (Eggset et al., 1983; Eggset et al., 1984). However the thin upper layer of epidermal keratinocytes is highly susceptible to the genotoxic effects of UV (Eggset et al., 1983). Thus, epidermal keratinocytes are the relevant cells in the skin for studies with respect to the induction and removal of UV-induced pyrimidine dimers in vivo.

Unfortunately, most studies on dimer removal in mammalian cells, including our own (Vijg et al., 1984), have been performed with cultured fibroblasts. In cultured rodent fibroblasts, UV-induced pyrimidine dimers are rather persistent. It was unknown, however, whether this well documented persistency would also be a characteristic of cultured keratinocytes. In view of the intended comparison between the in vivo and the in vitro situation we were obliged to first address this problem.

For an optimal comparison of DNA repair in fibroblasts with that in keratinocytes, these cells were separately isolated from a rat skin biopsy and subsequently metabolically labeled with ^{3}H-thymidine and ^{14}C-thymidine, respectively. Figure 2.1 clearly shows that there is no difference in dimer removal between fibroblasts and keratinocytes after irradiation in vitro with UV-B. In neither of the two cell types was there any significant repair over the first 3 h, while after 24 h only 20% of the dimers was removed. These DNA repair time courses were completely identical to that found for rat

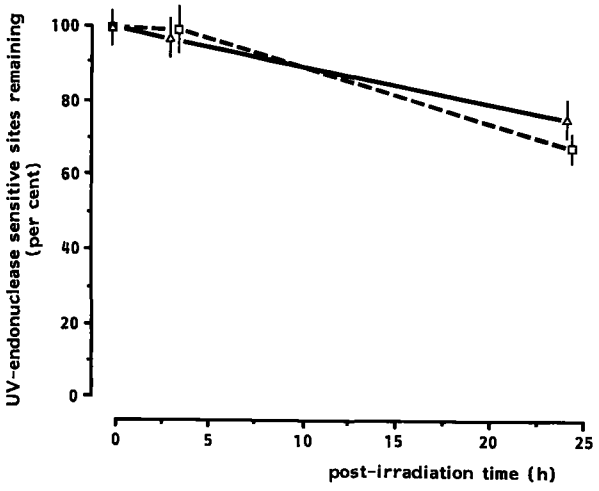

Figure 2.1: Time course of the removal of ESS from the DNA of rat fibroblasts (Δ) and epidermal keratinocytes (□) in vitro after irradiation with 4,000 J/m^2 of UV-B. Under the experimental circumstances such a dose induces about 180 ESS/10^9 molecular weight (mw) of DNA. Each point is the mean of 3 determinations. Bars represent the standard deviations.

fibroblasts after irradiation with UV-C (Vijg et al., 1984). The lack of difference in repair between keratinocytes and fibroblasts is in agreement with the results of Taichman and Setlow with cultured human cells (Taichman and Setlow, 1979).

For the determination of pyrimidine dimers in the DNA of epidermal keratinocytes irradiated in vivo with UV-B we used our recent modification of the UV-endonuclease assay (Vijg et al., 1986b). We have shown that for rat fibroblasts radioactivity gradient profiles coincide well with immunochemical ones (Vijg et al., 1986b). Furthermore, dose response and DNA repair time course studies on UV-irradiated radioactive and non-radioactive rat fibroblasts using the original assay and our modified method, respectively, yielded identical results (unpublished data). As shown in Fig. 2.2, in this present study a linear dose dependency was found when epidermal keratinocytes were irradiated in vivo with UV-B. The detection limit was about 500 J/m^2 of UV-B, which is relevant for human exposures as it is the equivalent of 1-2 times the minimal erythemal dose (Eggset et al., 1983).

Subsequently, we determined the number of pyrimidine dimers in rat

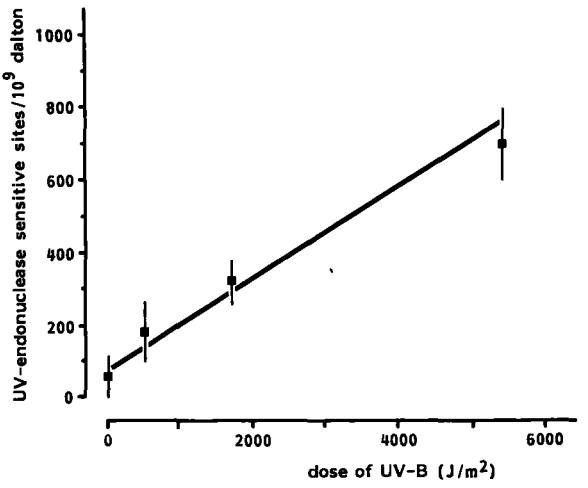

Figure 2.2: Dose response curve for the induction of ESS by UV-B in epidermal keratinocytes in vivo. Points indicate the mean of determinations on 3 different rats. Bars represent the standard deviations.

epidermal skin DNA, immediately after exposure to 4,000 J/m^2 of UV-B and at 3 and 24 h thereafter. During the repair periods the animals were kept in the dark. After 3 h of repair, a substantial shift of the DNA distribution in the sucrose gradient towards a higher molecular weight was seen (Fig. 2.3),

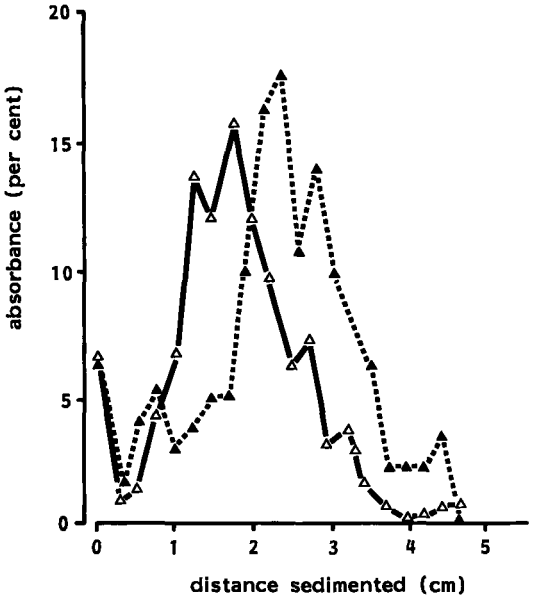

Figure 2.3: Profiles of rat skin DNA after treatment with M. luteus extract and centrifugation in alkaline sucrose gradients. DNAs were isolated from the epidermis of the skin biopts at 0 h (△) and at 3 h (▲) after irradiation in vivo with 4,000 J/m^2 UV-B. This dose induces about 550 ESS/10^9 mw of DNA (see Fig. 2.2).

which clearly demonstrated that rat epidermal keratinocytes in vivo are well able to remove pyrimidine dimers over this short time interval. The increase in molecular weight corresponds to 50-60 % removal.

Figure 2.4 shows the kinetics of pyrimidine dimer removal in epidermal cells in vivo after irradiation with UV-B. Although the removal of pyrimidine dimers in the epidermis was initially rapid, about 30% of the dimers originally induced were still present at 24 h after irradiation (Fig. 2.4). The persistency of a substantial fraction of UV-induced pyrimidine dimers in rat skin was confirmed by a non-quantitative immunofluorescence assay using a monoclonal

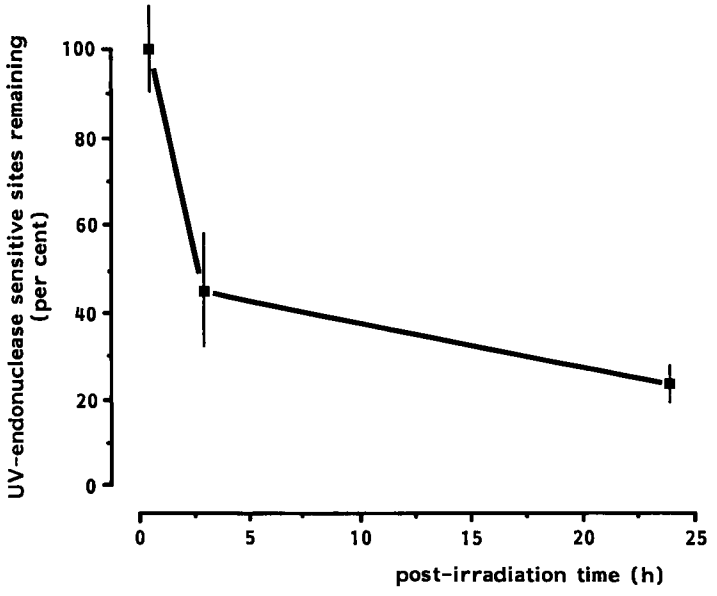

Figure 2.4: Time course of in vivo repair of ESS in rat skin after 4,000 J/m^2 of UV-B. Each point is the mean of determinations (in triplicate) on 4 different rats. Bars represent the standard deviations.

antibody against pyrimidine dimers (Vijg et al., 1987; Mullaart et al., 1989a, Chapter 3); 48 h after irradiation low levels of fluorescence were still observed in the nuclei of epidermal cells in cryostat sections (Mullaart et al., 1989a; Chapter 3).

2.4 Discussion

The results described above indicate that rat epidermal keratinocytes in vivo are able to remove 50-60 % of UV-induced dimers by dark repair within 3 h, whereas in the same cell type in vitro no significant removal occurs over this time period. Although we are not aware of any previous studies in which such a direct comparison has been made, this result is not completely unexpected. Peleg et al. (1977) and La Belle and Linn (1984) showed that early embryonic mouse cells have the capacity to remove UV-induced pyrimidine dimers; this capacity was found to be lost after a few passages in vitro. In addition, data from Sutherland et al. (1980) and D'Ambrosio et al. (1981) indicate a more rapid repair of dimers in human skin cells in vivo than that found by numerous others, including ourselves (Vijg et al., 1984; Zelle and Lohman, 1979), for human fibroblasts in culture.

On the basis of our present results we may now definitely conclude that the capacity of rat skin cells to remove pyrimidine dimers is almost completely lost upon transfer of these cells into culture. It is not clear, however, whether the loss of the capacity for dimer removal has serious consequences for cell survival or preservation of genetic integrity. In spite of their much lower dimer-removing capability, rat fibroblasts in vitro have the same survival curve after UV-irradiation as cultured human fibroblasts (Vijg, 1987). Therefore the question arises whether rodent cells in culture rely more heavily on other DNA repair pathways, such as post-replication repair, than human cells under the same circumstances. On the basis of these considerations we suggest that the rat skin cells used in our study switch from one DNA repair pathway to

another during their transfer into culture, possibly in response to the greatly altered circumstances these cells have to deal with during active replication in vitro, as compared to the relatively "resting" state in the skin.

A major implication of our present findings is that one should be cautious with the extrapolation of data on DNA repair responses, obtained with cultured cells, to the living organism.

CHAPTER 3

THE REMOVAL OF UV-INDUCED PYRIMIDINE DIMERS FROM DNA OF RAT SKIN CELLS IN VITRO AND IN VIVO IN RELATION TO AGING [1]

3.1 Introduction

Ultraviolet light (UV)-induced DNA damage is considered to play an important role in the etiology of human skin cancer (Setlow, 1978). This is illustrated by the dramatic increase in the amount of UV-induced skin cancers in patients with xeroderma pigmentosum (XP), the cells of which have a reduced capacity to remove UV-induced pyrimidine dimers (Robbins et al., 1974). In addition to skin cancer, also skin aging might be causally related to DNA damage induced by the ultraviolet portion of sunlight. Although there is no direct evidence for such a relationship, this possibility is supported by the observation that the survival of skin cells after UV-irradiation, as well as their in vitro lifespan, is diminished when they are derived from sun-exposed areas of the skin as compared to cells from parts that are mostly protected from sunlight (Gilchrest, 1979; Liu et al., 1985).

Upon aging an accumulation of UV-induced DNA damage in skin cells might occur, as the result of an age-related decrease in the efficiency of the cellular systems for DNA repair. On the other hand, also when the capability to repair DNA damage remains constant during aging damage might accumulate gradually due to incomplete repair (Gensler and Bernstein, 1981; Tice and Setlow, 1985). Substantial data are available on the possible relationship between the extent of UV-induced DNA repair synthesis in cultured cells and the age of the donor. Both Nette et al. (1984) and Kempf et al. (1984), using human epidermal

[1] This work has been published previously by Mullaart et al., 1989a.

keratinocytes and mouse fibroblasts, respectively, found an age-related decrease in the level of UV-induced DNA repair synthesis. However, Hennis et al. (1981) and Liu et al. (1982), using human fibroblasts and keratinocytes, respectively, found no such age-related decline. Ishikawa and Sakurai (1986) found an age-related decrease in the level of unscheduled DNA synthesis (UDS) in mouse keratinocytes in vivo, but only after high UV doses. Results from our laboratory indicated a decrease of about 18 % in the amount of UV-induced UDS in cultured skin fibroblasts from old rats relative to those from young animals (Vijg et al., 1985). However, terminally differentiated cells are generally characterized by low DNA repair capacities (Vijg et al., 1986a). Therefore, the possibility exists that the small age-related decrease in UDS actually reflects a slight increase in the rate at which fibroblasts from old rats differentiate upon culturing in vitro (Vijg et al., 1986a). The relevance of cellular factors such as differentiation state in studies on DNA repair activities in fibroblasts in relation to donor age has been discussed earlier (Turturro and Hart, 1984).

The discrepancies between the studies mentioned above, might be partly explained by the fact that all authors studied the synthesis step in the excision repair process, by measurement of the incorporation of radioactively labeled thymidine. This incorporation, however, can be influenced by many factors such as cellular membrane transport, intra-cellular pool size of thymidine and thymidine-kinase activity. Therefore, a more reliable approach in the study of UV-induced damage and its repair is to monitor the presence of the damage itself, which makes it possible to determine the amount of damage induced and its persistency. With the exception of a study by Hall et al. (1982), who found no age-dependency in the ability of cells to repair damage in UV-irradiated infecting viruses, no information is available about the age-dependency of the removal of the UV-damage itself.

The aim of the present study is to monitor the possible age-related changes in the removal of the most abundant and important UV-induced DNA damage, i.e. the pyrimidine dimer, in fibroblasts in vitro and epidermal keratinocytes

in vivo. Within the detection limit of the technique used, no age-related differences could be observed, neither in vitro nor in vivo. However, the observation that a considerable part of the pyrimidine dimers induced are persistent, in both situations, supports the hypothesis that continuous exposure to genotoxic agents may lead to an accumulation of DNA damage with age.

3.2 Materials and Methods

3.2.1 Animals

The animals used were 6- and 36-month old female WAG/Rij rats, present in the aging colony at the TNO Institute for Experimental Gerontology; they were well defined in terms of age-related pathology, survival and health status (Burek, 1978; Van Zwieten, 1984).

3.2.2 Cell isolation and culture

Rat fibroblasts were obtained from skin biopsies of young and old rats as described earlier (Vijg et al., 1984). The cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal calf serum and antibiotics. The cells were used between passage 5 and 10. Human skin fibroblasts, obtained in the same way from skin biopsies from normal young individuals (20-30 years old), were used between passage 15 and 25. Both rat and human fibroblasts were used as an exponentially dividing population.

3.2.3 Determination of pyrimidine dimers in fibroblasts in vitro.

Fibroblasts were labeled with ^{3}H-thymidine (1 μCi/ml, 25 Ci/mmol, Amersham) for 3 days and subsequently cultured for 1 day in fresh non-radioactive medium.

The cells were washed with phosphate buffered saline (PBS) containing 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.6 mM KCl and irradiated with UV-C (254 nm) emitted by a low pressure mercury vapour lamp (Philips TUV, 15 W) at a fluence rate of 0.38 W/m^2. After irradiation, the cells were incubated with fresh medium for 0-, 3-, 7- and 24-h periods, washed with PBS and immediately frozen on dry ice. The number of dimers was determined, as the number of Micrococcus luteus UV-endonuclease sensitive sites (ESS), by alkaline sucrose gradient centrifugation, as described in Chapter 2.

3.2.4 Determination of pyrimidine dimers in epidermal keratinocytes in vivo.

The number of ESS (pyrimidine dimers) in the DNA of skin epidermal cells upon irradiation in vivo was determined with our recently modified UV-endonuclease assay (Vijg et al., 1986b; Mullaart et al., 1988a; Chapter 2). Skin biopsies were taken at 0, 3 and 24 h after irradiation with UV-B (290-320 nm) emitted by a Philips TL-20/12 fluorescent sunlamp, at a fluence rate of 11 W/m^2. During the repair period the animals were kept in the dark to exclude the possibility of photoreactivation repair.

3.3.5 Immunohistochemical determination of pyrimidine dimers in vivo.

A narcotized rat was shaved and irradiated with UV-B as described above. A biopsy was taken and directly frozen in liquid nitrogen. Sections (5 μm) were made on a cryomicrotome (Minotome, Int. Equipment Company). The tissue was fixed for 5 min in 70 % ethanol, followed by incubation for 2 min in 0.07 M NaOH in 70 % ethanol. Then the tissue was incubated for 1 h at 37°C with a mouse monoclonal antibody against thymine dimers (Roza et al., 1988), followed by an incubation for 1 h at 37°C with fluorescein-labeled goat anti-mouse IgG antibodies (Nordic, Tilburg, The Netherlands). To localize the nuclei, the same cryostat section was also stained with propidium iodide (0.03 μg/ml, in PBS).

The slides were mounted in glycerol/PBS (9:1) pH 8.0, containing 2.3 % 1,4-Diazobicyclo[2.2.2]octane (DABCO, Sigma) to reduce photo-bleaching, and examined with an Orthoplan fluorescence micro-scope (Leitz, Wetzlar, FRG), equipped with a Ploemopak epi-illuminator and with BP 450-490, KP 560, LP 515 filters (fluorescein) and BP 546/14, LP 580 filters (propidium iodide). Photographs were taken with a Leitz Orthomat on 35 mm 200 ASA Kodak Ektachrome film (Kodak Eastman, Rochester, NY).

3.3 Results

Pyrimidine dimers in DNA can be detected with the M.luteus UV-endonuclease, which nicks the DNA strand next to a dimer (Paterson et al., 1973). The amount of breaks (UV-endonuclease sensitive sites (ESS)), can then be determined by centrifugation in alkaline sucrose gradients (Fig. 3.1). With this assay the removal

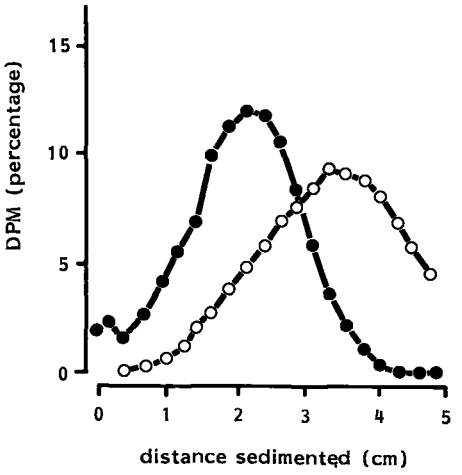

Figure 3.1: Alkaline sucrose gradient profiles of DNA isolated from rat fibroblasts directly upon irradiation with 4.6 J/m^2 UV-C after treatment with the M. Luteus extract (●) or without treatment (o).

of ESS in cultured fibroblasts isolated from young and old rats, irradiated with 4.6 J/m^2 UV-C, which induces about 180 ESS per 10^9 molecular weight (mw) of DNA, was determined, in comparison to the removal in human fibroblasts

irradiated with the same dose of UV (Fig. 3.2). As demonstrated previously, fibroblasts isolated from young rats consistently remove about 20 % of the dimers within a repair period of 24 h, whereas the human cells remove about 70 %

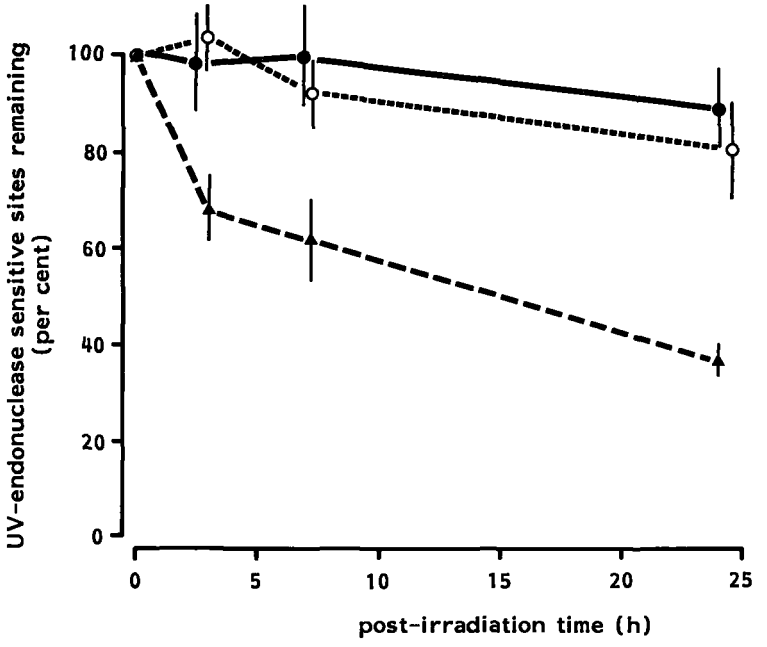

Figure 3.2: Time course of the removal of ESS from the DNA of fibroblasts isolated from young rats (6 months;o) and old rats (36 months;●) and from cultured human fibroblasts (▲) after irradiation with 4.6 J/m^2 UV-C (254-nm UV). Each point is the mean of determinations (in triplicate) on two different rat or human fibroblast cell lines. Bars represent the standard deviation.

during this period (Vijg et al., 1984). Interestingly, the fibroblasts isolated from 36-month old rats show the same pattern of removal as those derived from young rats (Fig. 3.2). Thus, these results do not indicate a decline of the repair capacity of fibroblasts with age.

The necessity of studying DNA repair processes not only in cultured cells, but also in the intact animal, was stressed by our earlier results which indicated that both rat fibroblasts and keratinocytes in vitro are substantially less proficient in removing pyrimidine dimers than epidermal keratinocytes in vivo, which remove about 50 % within 3 h (Mullaart et al., 1988a; Chapter 2). Therefore, we also determined the removal of pyrimidine dimers from rat epidermal keratinocytes in vivo in relation to donor age. Since UV-C hardly penetrates the skin, we used UV-B for the irradiation of the epidermal skin cells (Kodamo et al., 1984). Figure 3.3 shows the removal of ESS in vivo in epidermal keratinocytes from young

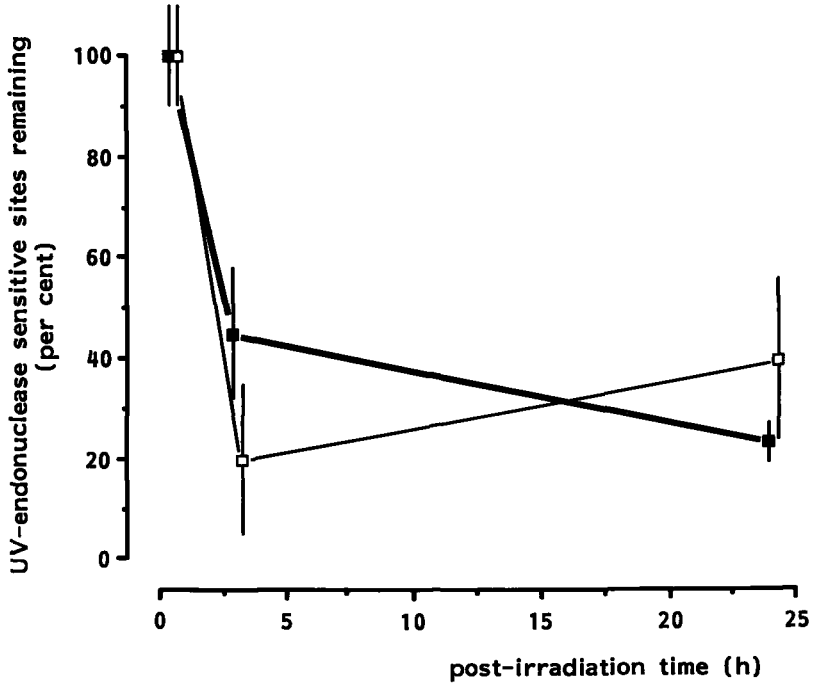

Figure 3.3: Time course of in vivo removal of ESS from the DNA of epidermal skin cells from young (■) and old (□) rats after irradiation with 4,000 J/m^2 UV-B (290-320 nm). Each point is the mean of determinations (in triplicate) on 3 different rats. Bars represent the standard deviation.

and old rats, after irradiation with 4,000 J/m^2 UV-B, which induces about 550 ESS per 10^9 mw of DNA. These results make it clear that the pattern of removal, a rapid initial phase followed by a much slower one, is not significantly different between keratinocytes from young and old rats.

The results presented above indicate that there are no considerable age-related changes in the extent of pyrimidine dimer removal, neither when tested in vitro nor in vivo. However, a substantial fraction of the pyrimidine dimers appeared to be rather persistent, both in fibroblasts in vitro and in rat epidermal keratinocytes in vivo. With cultured fibroblasts, this persistency was also indicated by HPLC analysis (Vijg, 1987). For the in vivo situation, persistency of pyrimidine dimers was confirmed by a specific non-quantitative immunofluorescence assay, based on the use of an anti-thymine dimer antibody (Fig. 3.4). The DNA in the

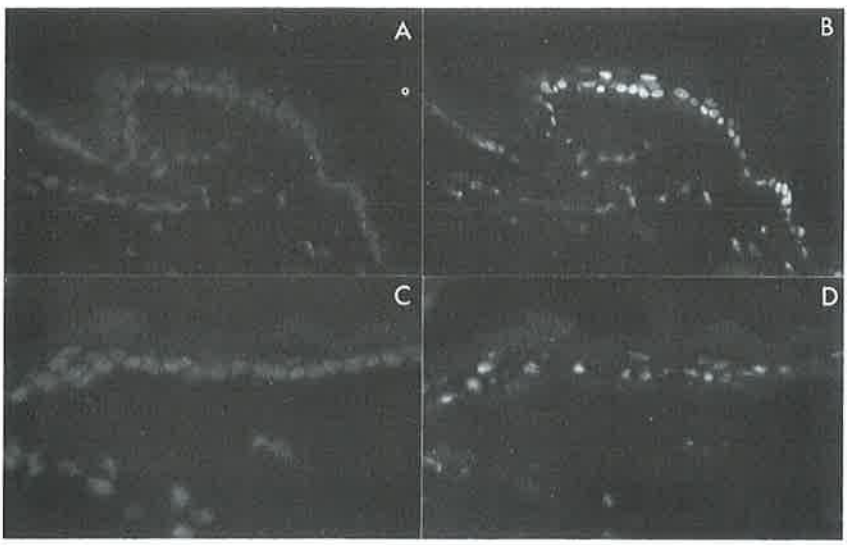

Figure 3.4: Immunohistochemical detection of thymine dimers in cryostat sections from the skin of a young rat, irradiated with 2,600 J/m^2 UV-B. The sections were incubated with an antibody against thymine dimers, the binding of which was detected with a fluorescein-labeled second antibody (B,D), and then stained with propidium iodide to visualize DNA in the nuclei (A,C). Skin biopsies were taken directly 0 h (A,B) and 48 h after irradiation (C,D). Magnification: 50x.

nuclei of epidermal keratinocytes and fibroblasts was visualized by staining with propidium iodide (Fig. 3.4A,C). Immunostaining performed directly after irradiation with 2,600 J/m^2 UV-B, revealed bright nuclear fluorescence in the thin upper layer of epidermal cells, while the fibroblasts, located deeper in the skin, were only weakly fluorescent (Fig. 3.4B). No fluorescence was observed in unirradiated skin samples (results not shown). After an in vivo repair period as long as 48 h, the fluorescence in the nuclei of the epidermal cells was lower, but still clearly visible (Fig. 3.4D).

3.4 Discussion

Proficient DNA repair is considered to be an important defense system against the deteriorative effects that threaten cells during aging, since it would protect cellular DNA from accumulating DNA damage and its consequences, such as gene mutations (Tice and Setlow, 1985). In this study we have investigated the capacity of skin cells from young and old rats to remove low levels of UV-induced pyrimidine dimers from their DNA in vitro and in vivo. The use of low UV-doses to study DNA repair processes in relation to age is preferable since high doses might inhibit the repair in an age-related manner. Indeed, earlier results from our laboratory indicated that in vitro a UV dose of 10 J/m^2 inhibits the repair process (Vijg et al., 1984). However, the method is not sensitive enough to monitor dimer removal after UV-C doses lower than 4.6 J/m^2.

In vivo the situation is much different. The sometimes large variation (upto 50%, see Fig. 3.3) made it necessary to use a dose of 4,000 J/m^2 in order to accurately quantify pyrimidine dimer load. However, we want to stress that this is still a low dose which does not induce severe cellular deterioration. Extrapolated to the human situation this UV dose is equivalent to 6-10 minimal erythemal doses (Ishikawa and Sukurai, 1986).

The major conclusion is that neither in vitro nor in vivo substantial age-related differences in pyrimidine dimer removal in rat fibroblasts and epidermal

keratinocytes appear to exist. During a repair period of 24 h, cultured fibroblasts isolated from both young and old rats removed 20 % of the pyrimidine dimers originally induced. Epidermal keratinocytes in vivo from old rats are, like those from young rats, well capable to rapidly remove at least 50 % of the pyrimidine dimers within 3 h.

As demonstrated earlier (Mullaart et al., 1988a; Chapter 2), the difference in removal between fibroblasts in vitro and epidermal keratinocytes in vivo cannot be attributed to a difference in cell type, as rat keratinocytes in vitro are comparable to cultured fibroblasts with respect to dimer removal. It should be realized that the method used for the determination of pyrimidine dimers in vitro, only measures repair in replicating cells which can incorporate ^{3}H-thymidine. Possible age-related changes in non-replicating cells will not be detected. However, previous results from our laboratory indicated, that more than 70 % of the fibroblasts from both young and old animals incorporate ^{3}H-thymidine within 24 h as checked by autoradiography (Vijg et al., 1986a).

Although the removal of pyrimidine dimers in vivo is very rapid initially, 24 h after irradiation about 30 % of the dimers induced are still present. Our additional results, i.e. those obtained with specific antibodies against thymine dimers, show that in vivo as late as 48 h after irradiation, a considerable part of the damage is still detectable. In spite of the lower number of pyrimidine dimers induced, this persistency is even more pronounced in rat fibroblasts in vitro: 24 h after irradiation 80 % still remained (Fig. 3.2). Indeed, results obtained with confluent fibroblasts indicated only about 30 % removal at 5 days after irradiation (results not shown). As yet, no information is available about the level of dimer removal in fibroblasts in vivo. At the moment the immunohistochemical assay is further improved to allow quantitative measurements of low levels of pyrimidine dimers in vivo (Roza et al., 1988).

In cultured human fibroblasts about 10 % of the pyrimidine dimers is still present 5 days after irradiation, and only after 10-20 days dimers no longer can be detected (Kantor and Setlow, 1981). In human skin cells too, dimer removal

proceeds much more rapidly in vivo than in vitro; nevertheless, 24 h after irradiation in vivo about 10 % of the dimers are still present (D'Ambrosio et al., 1981).

This raises the question whether there might be an age-related accumulation of pyrimidine dimers in skin cells, when frequently exposed to UV. In rats such an accumulation can not be expected since they are not exposed to UV under normal circumstances. Indeed, our results indicate that the background level of dimers in both young and old rats was never higher than a few dimers per 10^9 mw of DNA. However, data from Gilchrest et al. (1979) and Liu et al. (1985) suggest that continuous sun-exposure can indeed damage human skin cells. They found a marked increase in sensitivity to UV-C, with respect to effects on growth and survival, in cultured cells isolated from sun-exposed skin areas from aged human donors compared to cells from areas of the skin from the same individuals that are mostly protected from sunlight. In view of these results it would be interesting to screen skin biopsies, for example by using the anti-thymine dimer antibody, from donors of different ages and at different levels of sun-exposure for the presence of accumulated pyrimidine dimers. In addition, there is an obvious need for further investigations into the influence of incomplete repair of DNA damage on processes such as aging and cancer in individuals continuously exposed to sunlight.

CHAPTER 4

AGE-RELATED INDUCTION AND DISAPPEARANCE OF CARCINOGEN-DNA-ADDUCTS IN LIVERS OF RATS EXPOSED TO LOW LEVELS OF 2-ACETYLAMINOFLUORENE [1]

4.1 Introduction

Exposure of the somatic cells of higher organisms to DNA-damaging agents has been suggested to underly both the induction of cancer and aging (Miller and Miller, 1974; Gensler and Bernstein, 1981). For cancer this possibility is supported by ample evidence (Lutz, 1979; Balmain, 1981). With respect to DNA damage and aging, the situation is much less clear. In part this is due to the fact that aging is a multifaceted process, which is not likely to be caused by one single factor. It is well conceivable, however, that the continuous exposure to low physiological levels of DNA-damaging agents is an important factor in the increased incidence of cancer with age. Several sources may contribute to the "natural" induction of DNA damage, such as radiation, body heat, food constituents, products of metabolism, etc. The effects of the DNA-damaging agents are counteracted by efficient enzymatic systems capable of repairing a wide range of DNA alterations. These systems are of the utmost importance for the survival of the cell, organism and individual, and probably for the mitigation of the aging process (Turturro and Hart, 1984; Vijg and Knook, 1987).

Thus far, the lack of sensitive techniques for the detection and quantitation of low levels of lesions in the DNA of various organs and tissues has precluded a direct study of DNA repair processes in vivo in relation to aging. Recently, biochemical and immunochemical techniques have been developed in several

[1] This work has been published previously by Mullaart et al., 1989b.

laboratories that allow accurate monitoring of the induction and removal of specific DNA lesions in old and young individuals exposed to low levels of genotoxic agents (for a review, see Vijg and Uitterlinden, 1987).

Here we report about the induction and disappearance of carcinogen-DNA-adducts in the liver of old and young rats after a single intra-peritoneal (i.p.) injection of a low dose of the model liver carcinogen 2-acetyl-aminofluorene (AAF). Adduct levels were quantitated at various timepoints after treatment by using a modified ^{32}P-postlabeling assay (Gupta, 1985). The results obtained indicate that the DNA lesions induced by a single low dose of AAF are rather persistent in both young and old rats. During the first day after treatment the induction of DNA-adducts was slower in old rats than in young animals, but after 2 days the adduct level was found to be higher in old rats. The disappearance of the AF-adducts was slightly slower in old rats as compared to young ones, resulting in a higher adduct level 21 days after treatment. These results are discussed in relation to the proposed role of spontaneous DNA damage in cancer and aging.

4.2 Materials and methods

4.2.1 Chemicals

2-Acetylaminofluorene (AAF) was purchased from Fluka AG. RNase A (50 U/mg) and RNase T1 (500,000 U/ml) were obtained from P-L Biochemicals. Micrococcal endonuclease (grade VI; 100 U/mg), Spleen exonuclease (18 U/mg), Potato apyrase (grade I; 2 U/mg) and spermidine free base were all obtained from Sigma. T4 polynucleotide kinase (50,000 U/mg) was from Boehringer. Deoxyribonucleotides were from P-L Biochemicals. N,N-bis-(2-hydroxyethyl)-glycine (bicine) and 1,4-dithiothreitol were obtained from Merck. Polyethyleneimine (PEI)-cellulose thin layers (20x20 cm) were from Merck (cat. no. 5579). Adenosine 5'-γ-^{32} P triphosphate with a specific activity

of 3,000 Ci/mmol (10 mCi/ml; purity: 98 %) was purchased from Amersham. The phase transfer agent tetrabutyl-ammonium chloride (TBA) was obtained from Aldrich Chemie. 1-Butanol (Merck) was saturated with deionized water prior to use.

4.2.2 Experimental animals and treatments

The animals used were 6- and 36-month old female Wistar-derived inbred rats (WAG/Rij) from the aging colony of the TNO Institute for Experimental Gerontology, kept under constant environmental conditions (Van Zwieten, 1984). Standard laboratory diet and were supplied ad libitum. The total bodyweight of the animals was about 180 ± 8 g for the young and 230 ± 12 g for the old ones and the liverweights were 4.9 ± 0.4 and 6.9 ± 0.6 g, respectively. No significant age-related changes in the ratio of body weight to liver weight were found. Rats were i.p. injected with single doses of 5-100 mg AAF per kg bodyweight in 0.2 ml of dimethyl sulfoxide. Control rats received dimethyl sulfoxide only. Animals were sacrificed at various timepoints after treatment by CO_2 anoxia. The livers were excised and kept frozen at -70°C until DNA isolation.

4.2.3 DNA isolation

Frozen livers were thawed in lysis buffer (5 ml per gram of liver). Lysis buffer was 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA. Proteinase K was added to 0.1 mg/ml and SDS to 1% (v/v), and the solutions were incubated at 37°C for 16 h while shaking gently. The solutions were extracted successively with equal volumes of phenol, (saturated with 10 mM Tris-HCl, pH 8.0) and chloroform:isoamylalcohol (24:1, v/v). The phases were separated by centrifugation at 6,000 g at 4°C for 15 min. DNA was precipitated from the final aqueous phase, after the addition of 0.1 volume of 3 M sodium acetate

(pH 5.6), with 2 volumes of absolute ethanol pre-cooled to -20°C. The precipitates were washed twice with 70% ethanol and dried at room temperature in a Speed Vac Concentrator (Savant Instruments). Subsequently, the DNAs were dissolved in 10 ml 10 mM Tris-HCl, pH 7.8, 1 mM EDTA. In order to remove RNA, all samples were incubated for 30 min with a mixture of RNase A (0.04 mg/ml) and RNase T1 (12 U/ml) at 37°C. After a 30-min incubation with Proteinase K (0.05 mg/ml) and SDS (0.1%, v/v), the samples were extracted once again with phenol and chloroform:isoamyl-alcohol as described above. DNA was precipitated from the aqueous phases and dissolved as described above. DNA concentrations were measured spectrophotometrically in a Perkin Elmer spectrophotometer. The ratios A_{260}:A_{280} and A_{230}:A_{260} were about 1.85 and 0.42, respectively. Usually about 3-3.5 mg of high molecular weight DNA (tested by electrophoresis in an 0.8% agarose gel) per rat liver was obtained. The DNA samples were kept frozen at -70°C.

4.2.4 ^{32}P-postlabeling assay

For the analysis of AAF-induced adducts in rat-liver DNA, the assay of Gupta et al. (1980) was used with slight modifications. Five µg DNA was digested with 1 U micrococcal endonuclease and 0.02 U spleen exonuclease in the presence of 10 µl 20 mM sodium succinate, pH 6.0, 10 mM $CaCl_2$, in a total volume of 30 µl at 37°C for 2½ h. HPLC analysis of the samples revealed that under these conditions the hydrolysis was complete; only the four mononucleotide peaks were observed. The digest was then diluted to 50 µl with deionized water.

To enrich for adduct-containing nucleotides, 10 µl of each digest (1 µg DNA) was mixed with 5 µl 100 mM ammonium formate, pH 3.5, 5 µl 10 mM TBA and 30 µl of water, and extracted twice with 1 volume of 1-butanol by agitating for 30 sec on a Vortex mixer. Phases were separated by centrifugation (1 min) in a tabletop microcentrifuge. In order to remove as many non-adducted

nucleotides as possible, the combined organic phases were re-extracted twice with 90 µl of water. The butanol phase (containing the adducted nucleotides) was neutralized by adding 1 µl of 200 mM Tris-HCl (pH 9.5) and evaporated in a Speed Vac Concentrator. The adduct residue from 1 µg of DNA was dissolved in 3 µl of water. To this solution a 7-µl aliquot of a freshly prepared radioactive mix was added. For 4 samples the radioactive mix was prepared as follows: 6 µl buffer mix (100 mM bicine, pH 9.0, 100 mM $MgCl_2$, 100 mM 1,4-dithiothreitol, 10 mM spermidine) was added to 2.3 µl of T4 polynucleotide kinase (10 U/µl) and 33.7 µl of water. From this solution 36 µl was added to 240 µCi freeze-dried γ-(^{32}P)ATP. The reaction mixture (the DNA and radioactive mix) was incubated for 30 min at 37°C.

For the purification and resolution of ^{32}P-adducts, the described "four-directional" two-dimensional PEI-cellulose thin layer chromatography (TLC) system was used (Gupta, 1985). Eight µl of the labeled adduct digests were applied at the origin. Conditions for the development were as described by Gupta (1985). Before autoradiography, the origin was excised from the PEI-cellulose sheet in order to remove as much background radioactivity as possible. In order to correct for variations in the efficiency of radioactive labeling, 5 µl of the original DNA digest was further diluted to 0.02 ng DNA/µl and 5 µl of this solution was evaporated in a Speed Vac Concentrator. To this total nucleotide residue 1 µl of the radioactive mix used for adduct-labeling was added. After incubation at 37°C, 1 µl of a solution containing 4 µg/µl of carrier dpAp, dpTp, dpGp and dpCp and 1 µl of Potato apyrase (40 mU/ml) were added and the incubation was continued for another 30 min. The labeled digest was then diluted to 50 µl with 10 mM Tris-HCl, pH 9.5, 5 mM EDTA. Five µl of the solution of ^{32}P-labeled total nucleotides were applied at 1.5 cm from the bottom edge of a PEI-cellulose layer (10 cm long). The sheet was developed to the top with 40 mM ammonium sulfate (Gupta, 1985).

Adducts were located by screen-enhanced autoradiography at -80°C for various time periods whereas total nucleotides were located by autoradiography at

room temperature for 15 min in Kodak XO-matic cassettes with 2 intensifying screens and Kodak XAR-5 films. Identification of adduct-spots was achieved by comparison with the synthetic adduct markers dG-C8-AAF and dG-C8-AF prepared as described by Kriek and Westra (1980). For the evaluation of radioactivity, spots were excised from the PEI-cellulose sheet and counted in glass scintillation vials without the addition of a scintillator (Cerenkov assay). Appropriate blank areas of the chromatograms were counted also and their count rates subtracted from those of the sample count areas. Since adducts were evaluated from 0.8 μg of DNA and total nucleotides were evaluated from 0.01 ng of DNA, the relative adduct level (RAL) was calculated as follows:

$$RAL = \frac{1}{80{,}000} \times \frac{\text{cpm in adducts}}{\text{cpm in total nucleotides}}$$

By multiplying RAL with 0.3×10^7, RAL values can be converted into fmol adducts per μg DNA, assuming 1 μg DNA = 0.3×10^7 fmol nucleotides:

$$\text{adduct level} = 37.5 \times \frac{\text{cpm in adducts}}{\text{cpm in total nucleotides}} \quad \text{fmol/}\mu\text{g DNA}$$

4.3 Results

Figure 4.1 illustrates the difference in sensitivity between the original ^{32}P-postlabeling procedure (Gupta et al., 1982), in which both the unmodified nucleotides and the adducted nucleotides are ^{32}P-labeled, and the recently published new procedure (Gupta, 1985), in which the adducted nucleotides are first separated from the unmodified nucleotides and subsequently ^{32}P-labeled. The two-dimensional chromatograms shown for both procedures

were obtained with liver DNA isolated from rats treated with 100 mg AAF per kg bodyweight and killed at 48 h after treatment. They indicate that with the old procedure one type of adduct, the aminofluorene (AF)-adduct, is detected and only after autoradiographical exposure of the chromatograms for a period of 5 days at -80°C (Fig. 4.1A). Obviously, the AF-adduct, the identity of which was established by comparison with dG-C8-AF, is the major adduct formed. With the new procedure also the acetylaminofluorene (AAF)-adduct was readily detected (Fig. 4.1B). Moreover, the time needed to detect the adduct-spots with autoradiography could be reduced to 1 day or less.

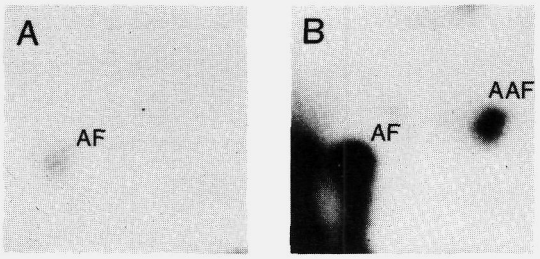

Figure 4.1: Two-dimensional chromatograms of digests of liver DNA from young rats treated with a single i.p. dose (100 mg/kg) of AAF. Animals were killed and DNA was extracted, 48 h after treatment, as described in Materials and Methods. Purification and resolution of ^{32}P-labeled adducts was performed with the original ^{32}P-postlabeling procedure (Gupta et al., 1982) or with the new procedure (Gupta, 1985) with modifications. (A) Original ^{32}P-postlabeling assay; autoradiographical exposure was for 5 days at -80°C. (B) New procedure; autoradiographical exposure was for 1 day at -80°C. After shorter exposures, the AF spot was found to be clearly separated from the unidentified background material below and to the left (results not shown). AAF = AAF-adduct; AF = AF-adduct.

The adducts formed after a dose of 100 mg/kg are easily detectable. However, this dose is toxic and higher than the level of exposure to natural carcinogens during normal aging. To establish whether AF- and/or AAF-adducts could still be detected after low doses, rats were treated with a 5- and 20-fold lower amount of AAF. Figure 4.2 shows the two-dimensional chromatograms of liver DNA, isolated from rats killed at 48 h after treatment with 100, 20 or 5 mg of AAF per kg bodyweight, respectively. The results indicate that at all doses the AF-adduct is the main adduct formed. While the AAF-adduct is also formed, it is clearly visible only at the two highest doses, 100 and 20 mg/kg (Fig. 4.2A and 4.2B, respectively); it was not detectable after the lowest dose, 5 mg/kg (Fig. 4.2C).

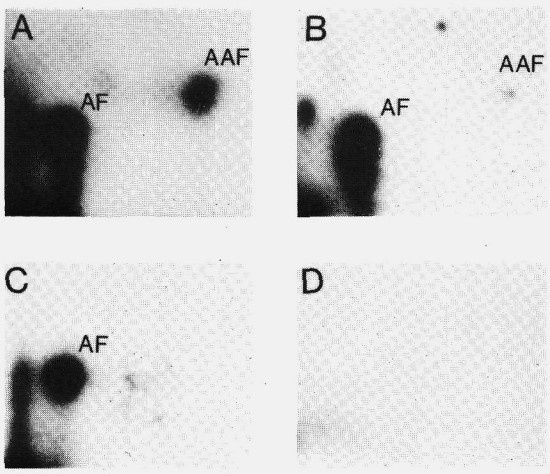

Figure 4.2: Two-dimensional chromatograms of digests of liver DNA from young rats treated with a single i.p. dose of 100 mg/kg (A), 20 mg/kg (B), 5 mg/kg (C) or 0 mg/kg (D) of AAF. After 48 h, the animals were killed and DNA was extracted; aromatic adducts were labeled and chromatographed as described in Materials and Methods. Autoradiographical exposure was for 3 days at -80°C. Prolonged exposure of the chromatograms did not reveal additional adduct-spots. AAF = AAF-adduct; AF = AF-adduct.

In further experiments, aimed at studying possible age-associated changes in the induction and disappearance of DNA-adducts at an acceptable level, rats were treated with 5 mg AAF per kg bodyweight. Figure 4.3 shows the

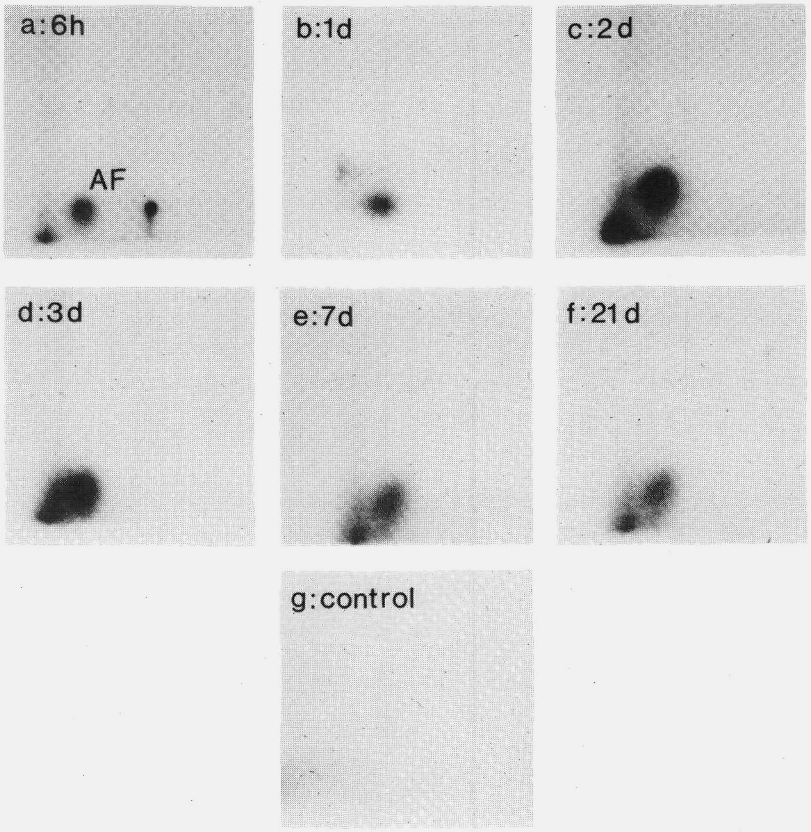

Figure 4.3: Two-dimensional chromatograms of digests of liver DNA from young rats treated with a single i.p. dose (5 mg/kg) of AAF. After the period indicated in the panels (A-F), the animals were killed; DNA was extracted and ^{32}P-fingerprinted as described in Materials and Methods. Autoradiographical exposure was for 10 h at -80°C. The additional spot in panel A was not observed in the other series of chromatograms and was therefore considered as background. The higher intensity of the AF spot at 6 h as compared to that at 1 day was due to the lower labeling efficiency of the DNA sample at that time point; this was taken into account in the calculation of adduct levels (see Materials and Methods). AF = AF-adduct.

two-dimensional chromatograms of liver DNA, isolated from 6-month old rats, killed at various timepoints after treatment with AAF. AF-adducts were already found to be present at 6 h after treatment. Between 6 and 48 h a sharp rise in the AF-adduct level was observed, whereas at 72 h the adduct-level was lower again (Fig. 4.3). This decrease continued until at 21 days after treatment the amount of adducts was about 20% of the 48-h level. More or less the same pattern was observed with old rats. However, after correction for variations in the efficiency of radioactive labeling (see Materials and Methods) it appeared that during the first day after treatment the induction of AF-adducts in old rats was slightly, though significantly, slower than that in young rats. This becomes apparent from Fig. 4.4, in which the amount of AF-adducts present in rat liver DNA of young and old rats at the various timepoints after treatment is shown. Whereas during the first day the rate

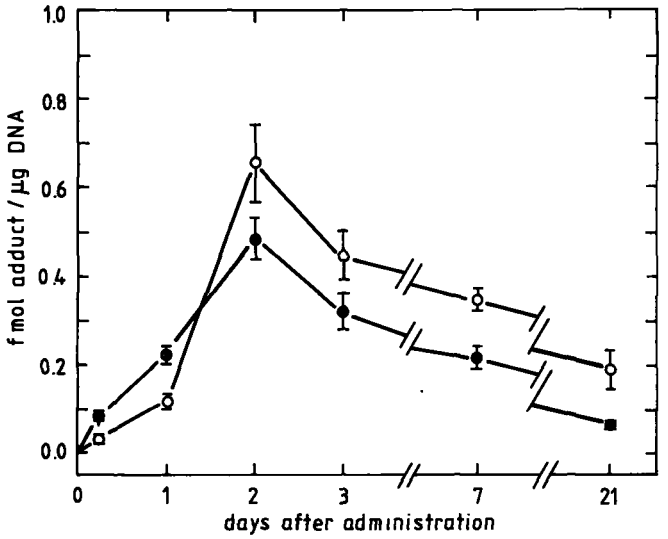

Figure 4.4: Rate of induction and disappearance of AF-adducts in rat liver following a single i.p. dose (5 mg/kg) of AAF in young (●) and old (o) rats. AF-adduct levels were measured at 6 h, 1 d, 2 d, 3 d, 7 d and 21 d. Each point represents a mean value from 3 rats. Bars indicate the experimental error (SD).

of induction of AF-adducts was found to be lower in old rats as compared to young rats, at 48 h after treatment the AF-adduct level in old rats was found to be significantly higher than that in young rats. The degree of modification at this time point was about 2.1 and 1.6 adducts per 10^7 nucleotides for young and old rats, respectively. The rate of disappearance of AF-adducts was found to be slow in both young and old rats. At 21 days after treatment, in old rats about 30 and in young rats about 20% of the 48-h level of AF-adducts was still present. When the adduct level was plotted on a semi-logarithmic scale, the disappearance of AF-adducts between 3 and 21 days appeared to be significantly slower in old rats than in young animals (Fig. 4.5). In absolute numbers, after 21 days the old animals had about twice the amount of persisting adducts than the young ones.

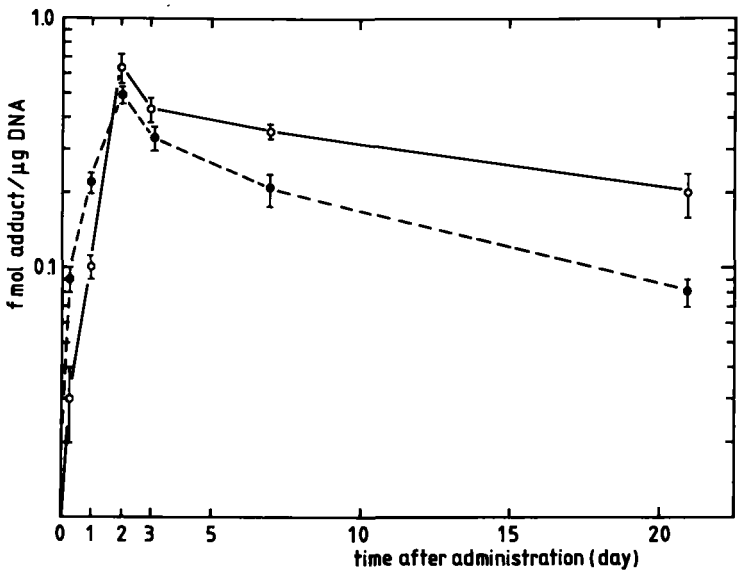

Figure 4.5: Rate of induction and disappearance of AF-adducts in young (●) and old (o) rats, plotted on a semi-logarithmic scale against the time after treatment. For details see legend Fig. 4.4. There is a statistically significant age-related difference ($P < 0.05$, as tested by Student's t-test) in the slopes of the curves representing the disappearance of AF-adducts between 3 and 21 days.

4.4 Discussion

Few studies have concerned themselves with the age-related induction and repair of carcinogen-DNA adducts in vivo. Results of Niedermüller et al. (1982; 1985) were interpreted in terms of a significant reduction in DNA repair capacity in aged rats after administration of various alkylating agents and treatment with ionizing radiation. However, the "old" animals used were not truly old, so that the results obtained did not cover the late stages of normal lifespan (Knook and Hollander, 1978). Moreover, these studies suffered from high doses and the use of radioactivity which could give rise to side effects and may interfere with the interpretation of the data in physiological terms (Ribeiro et al., 1985). In order to comparatively analyze DNA repair responses over large time intervals in animals in relation to age, low doses of nonradioactive test compounds are essential.

In the present study we have monitored the induction and disappearance of DNA-adducts in the rat liver in relation to age, after treatment with only one single dose of 5 mg per kg bodyweight of nonactivated, non-radioactive AAF. For this purpose we made use of the recently developed ultrasensitive ^{32}P-postlabeling assay (Gupta, 1985). By using this technique we showed that the AF-adduct is the main adduct formed in rat liver DNA after treatment with AAF (Fig. 4.1), which is in accordance with the literature (Stout, 1980; Aune et al., 1985; Gupta et al., 1985).

From the time-course study (Fig. 4.4) it can be concluded that in old rats the induction of AF-adducts during the first day is slightly slower as compared to the induction in young rats; nevertheless at 48 h after treatment, old rats have a higher level of AF-lesions in their DNA. In order to interpret this it should be realized that the amount of AF-adducts present in liver DNA at a certain moment depends on 2 processes, induction and disappearance. The first process involves the uptake of AAF by the blood, its transport to and uptake by the liver parenchymal cells and its subsequent metabolic

activation to reactive products that bind to DNA. The disappearance of induced carcinogen-DNA-adducts is thought to proceed predominantly via one or more DNA repair pathways. The slower induction of adducts in old rats, during the first day after treatment, can be explained in terms of age-related changes in the uptake, transport and metabolization of AAF; this is in keeping with the well documented age-related decrease in the clearance of many drugs which has been attributed to changes in liver physiology and composition (Van Bezooijen, 1984; 1986).

The higher adduct level at 48 h in old rats does not necessarily indicate that in liver cells of old rats more AF-adducts are induced in the genome than in liver cells of young rats. Old rats could reach a higher peak level because the disappearance of AF-adducts over the initial time period is slower than in young rats. Although between 3 and 21 days there is only a small age-related decrease in the rate of disappearance of AF-adducts, it is not inconceivable that initially this difference is much higher. In addition, it should be noted that the dose of AAF was adapted to the bodyweight (and thus also to the liverweight) and not to the number of cells. Results from De Leeuw et al. (1986), indicate that the age-related increase in liverweight is correlated to an increased mean cell volume; the number of parenchymal cells per whole liver remains the same. This would implicate that the amount of AAF administered per cell was higher for the old rats. Indeed, after correction for this higher dose, the same level of AF-adducts is found at 48 h in both young and old rats. However, the rate of induction and disappearance is still slower in old as compared to young rats (results not shown).

A general conclusion that can be drawn from our studies is that the AF-adduct has a persistent nature; AF-adducts were still present at 21 days after treatment (Fig. 4.4). This is in agreement with results from Visser and Westra (1981), who found that about 15 % of the AF-adducts was still present 30 days after treatment with a single dose of 3 mg radioactive AAF per kg bodyweight. In addition, our present results indicate that there are slight

age-related differences in the induction and disappearance of AF-adducts, culminating in a higher level of AF-adducts in liver DNA of old as compared to young rats at 21 days after treatment. These findings make it tempting to speculate that the age-associated elevation in tumor incidence may be attributable to the age-related accumulation of persistent lesions, for example as a result of the incomplete repair of "naturally occurring" DNA lesions. It is not inconceivable that, during the normal lifespan of an organism, there is an age-related accumulation of such lesions (see Mullaart et al., 1988b; Chapter 6).

CHAPTER 5

GENOTOXIC EFFECTS OF INTRAGASTRICALLY ADMINISTERED BENZO(A)PYRENE IN RAT LIVER AND INTESTINAL CELLS [1]

5.1 Introduction

Polycyclic aromatic hydrocarbons (PAH) are an important class of possible dietary carcinogens. They are present in various food compounds (Howard and Fazio, 1980) and are thought to be active through electrophilic intermediates generated during metabolic activation by cytochrome P-450 containing mixed function oxidases (Gelboin, 1980). Several of these metabolites are very reactive and can bind to various macromolecules in the cell, e.g. the DNA (Miller, 1978). The ability of benzo(a)pyrene (BaP) or its metabolites to interact with the DNA has often been indirectly tested with the UDS/hepatocyte test (Williams, 1977). The results obtained, however, are somewhat conflicting. Data from Michalopoulos et al. (1978) and Tong et al. (1981) indicate that BaP is able to induce UDS in rat hepatocytes, while data from Brouns et al. (1979) suggest that BaP is hardly, and then only after high doses, capable to induce UDS in cultured hepatocytes. These discrepancies might be due to differences in the functional state of the isolated cells (Lonati-Galligani et al., 1983).

During the metabolic conversion of PAHs, genotoxic products other than electrophilic ones are formed, such as free radicals (Ide et al., 1983; Lesko, 1984; Cerutti, 1985), which are capable of inducing single-strand breaks (SSB) and small base damages in DNA (Hariharan and Cerutti, 1972). Since virtually all B(a)p that enters the body via the gastro-intestinal tract, is metabolized in the liver and intestine (Laher et al., 1984), high levels of radicals might

[1] Parts of this work have been published previously by Mullaart et al., 1989c.

be present in these organs, which would be expected to induce substantial levels of DNA damage.

In this study we tested the ability of BaP to induce bulky adducts in liver DNA by determining unscheduled DNA synthesis (UDS) in hepatocytes of rats treated intragastrically with B(a)P. In addition, we tested for the presence of SSB and small base damages, by applying the alkaline elution assay on isolated liver and intestinal cells of the same rats. We found that BaP was unable to induce UDS in rat parenchymal liver cells in vivo, whereas a considerable number of SSB were found in the DNA of both parenchymal liver cells and intestinal cells, but not in that of non-parenchymal liver cells. The possibility is discussed that these sites were induced by radical intermediates, generated during the metabolization of BaP.

5.2 Materials and Methods

5.2.1 Animal treatment

The rats used were 6-month old female Brown Norway (BN/BiRij) rats. Benzo(a)pyrene (Sigma), 62.5 mg per kg bodyweight, was administered intragastrically as a solution in sunflower oil (1 ml at a concentration of 12.5 mg/ml (50 mM)). Control animals received sunflower oil only.

5.2.2 Cell isolation

Parenchymal liver cells were isolated by liver perfusion and incubation with collagenase (0.05%) as described in detail elsewhere (Brouwer et al., 1984). The non-parenchymal cells were isolated, after perfusion and incubation with collagenase, by density gradient centrifugation in Nycodenz as described previously (Hendriks et al., 1985). The viability of the cells, checked by trypan blue exclusion, was never less than 90 %, both from untreated and treated

animals.

Rat intestinal epithelial cells were isolated as described (Harrison and Webster, 1969; Webster and Harrison, 1969). Briefly, the small intestine was cut at both ends, thoroughly rinsed with ice-cold phosphate buffered saline (PBS) and subsequently slipped inside out over a 4-mm diameter stainless steel rod of a vibro-mixer (A.G. Für Chemie Apparatenbau, Zürich). After 3 min of pre-vibration in PBS, the epithelial cells were released by vibration for 30 min at a frequency of 50 Hz and an amplitude of 2 mm in NET buffer (130 mM NaCl, 5 mM EDTA and 10 mM Tris, pH 7.4). The viability of the cells was always more than 90 %, both from untreated and treated animals.

5.2.3 Unscheduled DNA synthesis

The amount of UDS in parenchymal liver cells was determined at 5 and 18 h after BaP administration. Within 1 h after the start of the perfusion, about $1x10^6$ cells, suspended in medium (Dulbecco's Modification of Eagle's Medium (DMEM) containing fetal calf serum (FCS) and antibiotics), were seeded onto cover slips placed in a 3.5-cm diameter petri dish and incubated for 2 h. After this period the non-attached cells were removed with the medium. The cells were then incubated for 17 h in fresh DMEM, containing FCS, antibiotics and 10 μCi ^{3}H-thymidine (25 Ci/mmol, Amersham). As positive controls, a number of dishes with cells from untreated rats were treated in vitro with the model genotoxic agents ultraviolet light (UV) and ethidium bromide (EB) and with BaP itself. Irradiation with UV (254-nm) was performed as described in Chapter 2. Incubation with EB and BaP, added to the medium as solutions in PBS and dimethylsulfoxide, respectively, was for 2 h at 37°C. After this treatment the cells were supplied with fresh medium containing ^{3}H-thymidine as described above. After an incubation period of 17 h, the cells were rinsed 3 times with medium without serum, incubated for 10 min with 0.1 M Na-citrate at room temperature and fixed in acetic acid/ethanol

(1/3). Autoradiographs were prepared as described earlier (Vijg et al., 1984). For each slide, the percentage of nuclear and cytoplasmic area occupied by silver grains was measured for at least 30 cells.

5.2.4 Alkaline elution

The amount of DNA breaks was measured by the alkaline elution method (Kohn et al., 1976; Stout and Becker 1982). About $5x10^5$ cells, suspended in 0.5 ml PBS, were added to 0.5 ml sarkosyl buffer (0.2 % sarkosyl, 2 M NaCl and 0.02 M EDTA, pH 10.0) layered onto a 25-mm diameter polycarbonate membrane filter (Nuclepore; pore size 5 μm). This lysis solution was removed by passage through the filter by gravity and replaced by an SDS lysis buffer (0.5% SDS, 0.01 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 8.0, and proteinase K (0.5 mg/ml)). After a 1-h lysis period at 20°C the solution was removed by gravity and the DNA on the filter was washed twice with 5 ml 0.02 M EDTA, pH 10. The DNA was eluted through the filter with 0.06 M NaOH, 0.02 M EDTA, pH 12.6 (calculated), at 30 μl/min. Six 4.5-ml fractions were collected. After the elution, the filter with the residual DNA was transferred to a glass vial and, after addition of 4.5 ml elution buffer, irradiated with 100 Gy of ^{60}Co-gamma-rays (Gamma-cell 100, Atomic Energy of Canada) in order to release the DNA. The six eluate fractions and the irradiated membrane fraction were neutralized with 0.8 ml 4 M NaCl, 0.6 M NaOH, 1.0 M NaH_2PO_4, which further contained Hoechst 33258 dye (0.5 mg/l). The fluorescence of the Hoechst/DNA complex was measured at 430 nm (filters 3-73, 4-76 and 5-58) by excitation at 370 nm (filters 7-54 and 7-60) in a Pye Unicam LC-FL detector. The elution results were plotted as the log percentage of DNA remaining on the filter as a function of the fraction number. Mean slopes of the linear initial part of the elution curves were used to calculate the number of DNA breaks.

To calibrate the alkaline elution assay, nuclei were irradiated (in PBS

in Eppendorf vials) at 0°C with a ^{60}Co-gamma source (Gamma-cell 200, Atomic Energy of Canada) at a dose rate of 8 Gy/min for 10 and 25 sec, respectively.

5.3 Results

A widely used assay for the genotoxic effects of carcinogens is the UDS/hepatocyte test (Williams, 1977). This test is based on the occurrence of nucleotide excision repair upon induction of bulky adducts; the resynthesis step in this repair process can be measured as UDS. The results obtained in this study (Table 5.1) indicate that BaP, added in vitro to parenchymal cells, was only positive at the extremely high doses of 25 and 50 mM. Both positive control agents UV and EB were able to induce a considerable amount

Table 5.1 UNSCHEDULED DNA SYNTHESIS IN RAT PARENCHYMAL LIVER CELLS

Agents	UDS[a]	
	nucleus	cytoplasm
A. In vitro		
None	2.3 ± 0.2[b]	3.1 ± 0.3
UV (5 J/m^2)	9.3 ± 0.7	2.8 ± 0.3
UV (10 J/m^2)	14.3 ± 0.9	3.6 ± 0.4
EB[c] (2.5 M)	8.9 ± 0.4	1.6 ± 0.2
BaP[d] (50 mM)	6.6 ± 0.6	2.6 ± 0.3
BaP (25 mM)	4.9 ± 0.5	2.3 ± 0.3
B. In vivo		
BaP (5 h post adm)	3.1 ± 0.6[e]	3.5 ± 0.7
BaP (18 h post adm)	1.9 ± 0.5	2.7 ± 0.2

[a] The amount of UDS is expressed as percentage of nuclear or cytoplasmic area occupied by silver grains.
[b] The experimental error (SEM) was estimated from determinations on 3 slides.
[c] Ethidium bromide
[d] Benzo(a)pyrene
[e] The mean of experiments performed on at least 2 rats (3 slides per rat) ± SEM.

of UDS. BaP failed to induce any UDS in vivo, that is, after intragastric administration, neither at 5 nor at 18 h (Table 5.1).

Radical-induced DNA damage, such as SSB and small base damages, can be detected by using the alkaline elution assay. With this assay SSB already present in the DNA, together with certain other lesions that are converted into SSB as a consequence of the exposure to alkali during the assay (alkali-labile sites) can be detected (Kohn et al., 1978). The method is based on the passage of DNA through pores of the membrane filter. In the alkaline medium used, double-stranded DNA unwinds, and the more SSB are present, the faster unwinding into single-stranded fragments occurs. The rate of elution, therefore, is a measure of the number of SSB.

Representative alkaline elution curves of rat parenchymal and non-parenchymal liver cells and of intestinal cells at 5 h after intragastric administration of BaP and after gamma-irradiation are shown in Figs. 5.1 and 5.2. The slope of such curves represents the rate of elution of the DNA

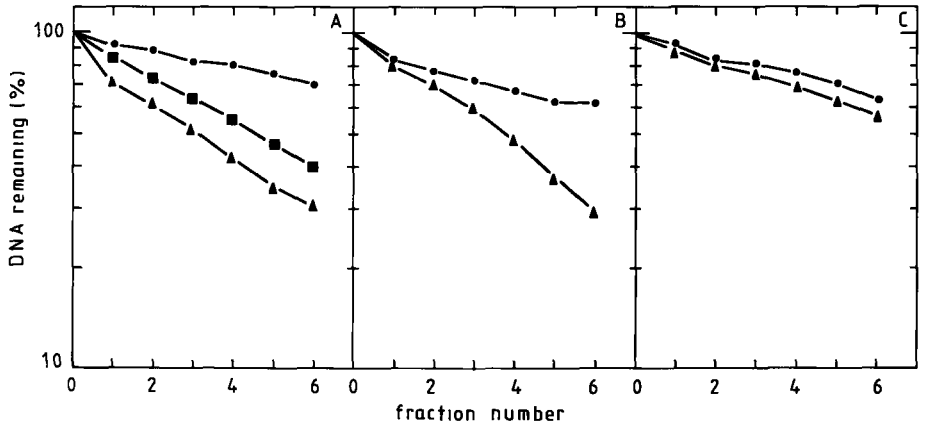

Figure 5.1: DNA alkaline elution curves of rat parenchymal and non-parenchymal liver cells at 5 h after administration of BaP. (A) parenchymal cells from an untreated rat after irradiation with 1 (■) or 2 (▲) Gy of gamma-rays or without irradiation (●). (B) parenchymal cells from an untreated (●) and a BaP-treated (▲) rat. (C) non-parenchymal cells from an untreated (●) and a BaP-treated (▲) rat.

through the membrane filter, which is a measure of the number of SSB plus alkali-labile sites. The elution curves obtained clearly indicate a higher level of SSB and alkali-labile sites in DNA of parenchymal liver cells (Fig. 5.1B) and intestinal cells (Fig. 5.2B) of the BaP treated animals as compared to those cells from untreated animals, while no such a difference was observed for the non-parenchymal liver cells (Fig. 5.1C). Gamma-rays, used as a positive control, were found capable of inducing SSB and/or alkali-labile sites in both liver parenchymal and intestinal cells (Figs. 5.1A and 5.2A).

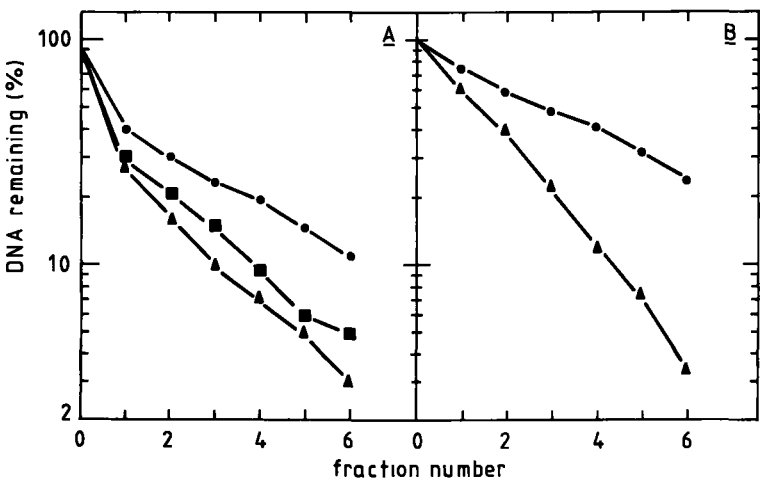

Figure 5.2: DNA alkaline elution curves of rat intestinal cells at 5 h after administration of BaP. (A) intestinal cells from the untreated rat after irradiation with 1 (■) or 2 (▲) Gy of gamma-rays or without irradiation (●). (B) intestinal cells from an untreated (●) and a BaP-treated (▲) rat.

The results from experiments performed on 4 different animals are summarized in Table 5.2. The exact number of SSB present in liver and intestine

DNA could be estimated by comparing the elution curves from these samples with those obtained with cells after gamma-irradiation. These data indicate that the number of BaP-induced SSB and/or alkali-labile sites is greater in intestinal cells than in parenchymal liver cells; as already shown in Fig. 5.1 no effect was found in the non-parenchymal liver cells.

Table 5.2 BAP-INDUCED ALKALI-LABILE SITES IN RAT LIVER AND INTESTINAL CELLS[a].

	Untreated	BaP treated
Parenchymal cells	0.25 ± 0.05[b]	0.50 ± 0.08
Non-parenchymal cells	0.30 ± 0.05	0.30 ± 0.05
Intestinal cells	0.55 ± 0.08	1.15 ± 0.25

a The numbers of alkali-labile sites induced by BaP were calculated from the elution profiles and expressed as SSB per 10^9 molecular weight (mw) of DNA. Calculations were made on the basis of gamma calibration curves, assuming that 1 Gy gamma-rays induces 0.25 SSB per 10^9 mw of DNA (Van der Schans et al., 1982).

b The experimental error (SEM) was estimated from 4 experiments on 4 different rats. Each experiment was performed in triplicate.

5.4 Discussion

The genotoxic effects of intragastrically administered BaP were studied in rat liver and intestinal cells. In liver cells in vivo, BaP was not found able to induce a significant amount of UDS. Even in vitro, after high doses the UDS response was found to be low. This lack of a UDS response in vivo might be due to the fact that there are no BaP bulky adducts formed at all. Indeed,

results using both the ^{32}P-postlabeling assay and immunochemical methods with specific antibodies against BaP adducts were all negative (Dr. R.A. Baan, personal communication).

In contrast, SSB and alkali-labile sites were found to be rapidly induced by BaP in parenchymal liver cells and in intestinal cells but not in non-parenchymal liver cells. Data from Lorentzen and T'so (1977) indicate that DNA breaks were induced in bacteriophage T7 DNA after treatment with BaP-diol. This metabolite is rapidly oxidized in the presence of O_2 and during this autoxidation radicals are generated, which can induce DNA damage. The fact that the formation of DNA breaks is reduced by the addition of superoxide dismutase (SOD), indicate that radicals are indeed responsible for the induction of DNA breaks. In addition, recent data from Leadon et al. (1988) demonstrate that also an other type of oxidative DNA damage, i.e. thymine glycol, is induced by BaP. Also in this case the formation of the damage could be inhibited by SOD, which indicate that radicals are responsible for the formation of thymine glycol. According to the data from Leadon et al. (1988) the total amount of oxygen radical-induced damage greatly exceeds the amount of bulky DNA adducts formed.

Therefore it is tempting to speculate that the SSB and alkali-labile sites found in parenchymal liver cells and in intestinal cells after in vivo treatment of a rat with BaP, are induced by radicals generated during the metabolization of BaP. The observation that these lesions were present in rat parenchymal liver cells and intestinal cells, the two major centers of metabolization, but not in the non-parenchymal liver cells, supports this hypothesis.

This lack of BaP induced alkali-labile sites in the non-parenchymal cells underlines the specific function of the parenchymal cells in the route of transportation and detoxification of BaP and many other genotoxic compounds present in the environment. Due to this specific function there might be a continuous induction of DNA damage in liver parenchymal cells during normal life, which might accumulate with age (see also Chapter 6).

In summary, we have shown that intragastric administration of BaP results in the formation of SSB and/or alkali-labile sites, while the lack of UDS suggests the absence of BaP DNA adducts. It should be mentioned that oxidative DNA damages (SSB and small base damages) are repaired via "short-patch" excision repair (Regan and Setlow, 1974), which will not be detected at all or only at a very low sensitivity by the UDS assay (Brouns et al., 1979). We suggest that these SSB and/or alkali-labile sites are induced via radicals generated during BaP metabolism. The absence of bulky DNA adducts might be the explanation for the fact that BaP is not a potent liver carcinogen in adult rats (Hsu et al., 1987). However, BaP might act as a tumor promotor agent by generating free radicals (Ide et al., 1983; Cerutti, 1985).

CHAPTER 6

AGE-DEPENDENT ACCUMULATION OF ALKALI-LABILE SITES IN DNA OF POST-MITOTIC BUT NOT IN THAT OF MITOTIC RAT LIVER CELLS [1]

6.1 Introduction

It has been suggested that aging is the result of an accumulation of DNA damage in post-mitotic somatic cells (Gensler and Bernstein, 1981). The rate of this accumulation could be determined by the activity of DNA repair systems of these cells, which may change with age (Gensler and Bernstein, 1981; Turturro and Hart, 1984).

A considerable amount of data is presently available with respect to alterations in the DNA structure in somatic tissue in relation to age (See Chapter 1). Price et al. (1971) used autoradiography to study the calf-thymus polymerase-catalyzed incorporation of labeled nucleotides into the DNA of neuronal nuclei of mice. More incorporation was found with 30-month old mice than with 3-month old mice. Since DNA polymerase requires 3'OH ends of DNA, this increased incorporation could represent breaks that have accumulated with age. This would be in agreement with the results of alkaline sucrose gradient centrifugation obtained by Ono et al. (1976) and Su et al. (1984), who found more single-strand breaks (SSB) in liver-cell DNA from 20-month old mice than in the DNA from 2-month old mice; the difference amounted to about $2\text{-}3 \times 10^4$ SSB per cell (60 - 150%). Massie et al. (1972a) found a decrease of the viscosity of rat liver DNA with age, which suggests a decrease in molecular weight of DNA. Using the S_1-nuclease digestion technique Chetsanga et al. (1975) found an age-related increase in the fraction of single-stranded DNA in rat liver of up to 25%.

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[1] This work has been published previously by Mullaart et al., 1988b.

By contrast, the results obtained by Dean and Cutler (1978), who used the same assay, but also thermal stability analysis and isopycnic density gradient centrifugation, gave no indication of an increase in the amount of single-stranded DNA. A disadvantage of several of the techniques used is their relatively low sensitivity, which does not permit the monitoring of low levels of DNA damage. In addition, DNA damage might be induced during processing of tissues and cells.

Thusfar, no data are available on DNA damage accumulation using sensitive techniques, in mitotic vs post-mitotic cells from the same organ. In the present study, we used a modified, highly sensitive alkaline elution assay, to quantitate alkali-labile sites in freshly isolated, non-radioactive DNA of post-mitotic and mitotic liver cells from young and old rats. We found that the DNA from post-mitotic parenchymal cells from old rats contains more alkali-labile sites than DNA from young rats. Such an age-related difference was not observed for the mitotically active non-parenchymal liver cells from young and old rats. These results are in keeping with the possibility that, even in the same organ, long-lived post-mitotic cells exhibit more pronounced symptoms of cellular senescence than actively dividing cells.

6.2 Materials and Methods

6.2.1 Animals

The animals used were 6- and 36-month old female Brown Norway (BN/BiRij) rats. They were kept under "clean conventional" conditions and fed a pelleted standard rodent diet (AM II, Hope Farms) (Van Zwieten, 1984). Both food and tap water were available ad libitum.

6.2.2 Cell isolation

Parenchymal liver cells were isolated by liver perfusion and incubation with collagenase (0.05%) as described in detail elsewhere (Brouwer et al., 1984). The non-parenchymal cells were isolated from the supernatant of the parenchymal cell suspension after centrifugation at 50 g for 2 min. After centrifugation (10 min, 350 g), the non-parenchymal cells were separated from erythrocytes and debris by density gradient centrifugation in Nycodenz as described previously (Hendriks et al., 1985). The viability of the cells, checked by trypan blue exclusion, was never less than 90 %.

6.2.3 Determination of DNA alkali-labile sites

Parenchymal and non-parenchymal cells were washed once with PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.6 mM KCl) and resuspended in PBS at a density of $1x10^6$ cells per ml. The amount of alkali-labile sites was measured by the alkaline elution method as described in Chapter 5.

The number of alkali-labile sites was determined in parenchymal and non-parenchymal liver cells from 3 young (6 months) and 3 old (36 months) rats. Measurements on a young and an old rat were always performed simultaneously to exclude artefacts of the cell isolation and alkaline elution procedure.

6.3 Results

The alkaline elution method is a highly sensitive assay for the detection of single-strand breaks (SSB) or lesions that are converted into SSB under alkaline conditions (Kohn et al., 1976). Until recently it was only possible to perform the alkaline elution assay on radioactively labeled DNA and therefore the method was restricted to cultured cells, which can be labeled during their S-phase with radioactive precursors involved in DNA synthesis. However, recent modifications

of this assay were introduced, which make it possible to determine alkali-labile sites in non-radioactive DNA of freshly isolated cells (Stout and Becker, 1982; Schutte et al., 1988).

Typical alkaline elution curves obtained with parenchymal liver cells - from a young rat - irradiated with different doses of gamma rays are shown in Fig. 6.1A. The slope of such curves represents the rate of elution of the DNA through the membrane filter, which is a measure of the number of SSB present or alkali-induced in the DNA applied to the filter. When these slopes were plotted against irradiation dose (Fig. 6.1B), a linear dependency was found for the induction

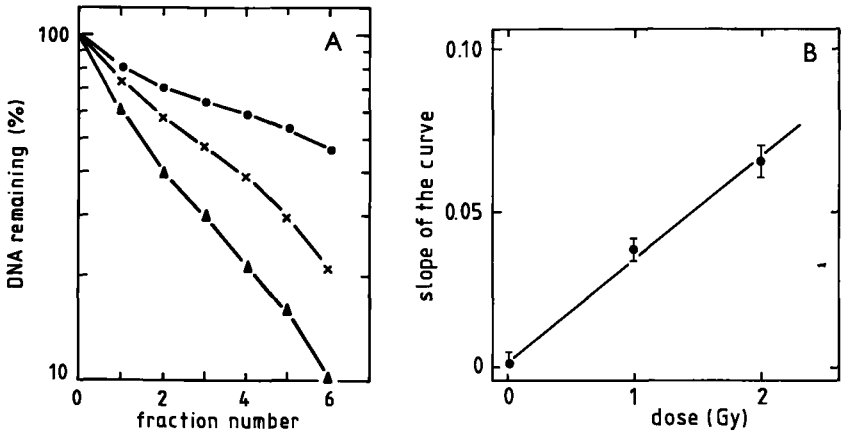

Figure 6.1: Induction of single-strand breaks (SSB) by ^{60}Co-gamma-rays in parenchymal liver cells from a young rat. (A) Alkaline elution of DNA from cells after irradiation with 0 (●), 1 (x) and 2 (▲) Gy. (B) Dose response of the induction of SSB by gamma irradiation. The number of SSB is expressed as the average slope of the elution curves of 3 independent determinations. The bars represent the estimated error (SD).

of SSB and alkali-labile sites. By extrapolation of classical results of sucrose gradient centrifugation it can be deduced that one Gy of gamma-irradiation induces about 0.25 SSB per 10^9 molecular weight (mw) of DNA (Van der Schans et al., 1982). As Fig. 6.1B shows, the alkaline elution technique used is sufficiently sensitive for the detection of SSB and/or alkali-labile sites present in unirradiated DNA. The method has a detection limit of about 0.06 sites per 10^9 mw of DNA, corresponding to a gamma-dose of 0.25 Gy. This limit is well below the number of sites determined in the unirradiated cells (Fig. 6.1A). Therefore, the method appeared very suitable for studying the low "physiological" levels of SSB and/or alkali-labile sites in DNA from young and old animals, to see whether any accumulation of these DNA damages occurs with age.

In these studies, the number of sites was determined in parenchymal and non-parenchymal liver cells from 3 young (6 months) and 3 old rats (36 months). In each experiment gamma-irradiations were included at 2 dosages, to calibrate the slopes of the alkaline elution graphs. Representative elution curves obtained with unirradiated parenchymal liver cells from a young and an old rat are presented in Fig. 6.2. The slopes are clearly different, the steeper slope belonging to the DNA isolated from the old rat. This indicates that the parenchymal cells from

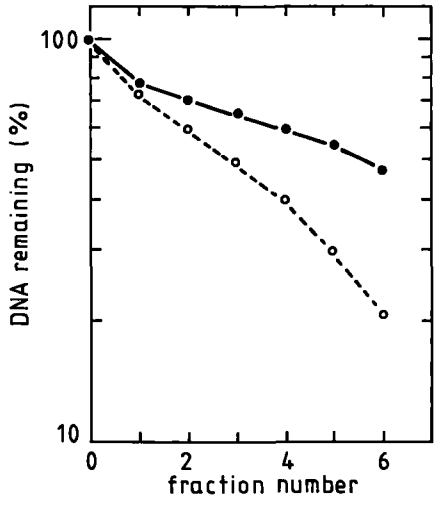

Figure 6.2: Alkaline elution of DNA from parenchymal liver cells isolated from a young (●) and an old (o) rat.

the old rat contain more DNA damage than such cells from a young rat.

The results of measurements on parenchymal cells from 3 young and 3 old rats are summarized in Table 6.1. The average numbers of sites detected via alkaline elution and their standard deviations are given. By using the gamma dose response curves for slope calibration, it was calculated that the DNA of parenchymal cells isolated from young rats contain about 0.22 detectable sites/10^9 mw of DNA, or 880 alkali-labile sites per cell (assuming a total mw of cellular DNA of $4x10^{12}$). The DNA of parenchymal cells from old rats however, contain 0.39 alkali-labile sites/10^9 mw of DNA (1,560 alkali-labile sites per cell). This difference is highly significant ($P < 10^{-4}$, when tested by variance analysis).

Table 6.1 NUMBER OF SITES DETECTED WITH ALKALINE ELUTION PER 10^9 MW OF DNA IN PARENCHYMAL AND NON-PARENCHYMAL LIVER CELLS FROM YOUNG AND OLD RATS.

Cell type	Age of the rat (month)	Experiment 1[a]	2[a]	3[a]	mean[b]
parenchymal	6	0.19±0.04	0.22±0.03	0.26±0.03	0.22±0.03
	36	0.40±0.07	0.38±0.08	0.39±0.04	0.39±0.04
non-paren-chymal	6	0.18±0.09	0.12±0.02	0.19±0.09	0.16±0.04
	36	0.21±0.01	0.10±0.03	0.20±0.03	0.17±0.06

[a] Derived from the mean slope of 3 alkaline elution curves of DNA from one rat ± SD.
[b] Mean of 3 different rats ± SD.

Interestingly, an age-related increase in the number of sites was not found in the non-parenchymal cell fraction isolated from the same livers. Figure 6.3 shows that the slopes of the elution curves are identical for the DNA of non-parenchymal cells isolated from young and old rats. Analysis of non-parenchymal cells from 3 young and 3 old rats, summarized in Table 6.1, indicates that there is no age-related difference in the number of sites.

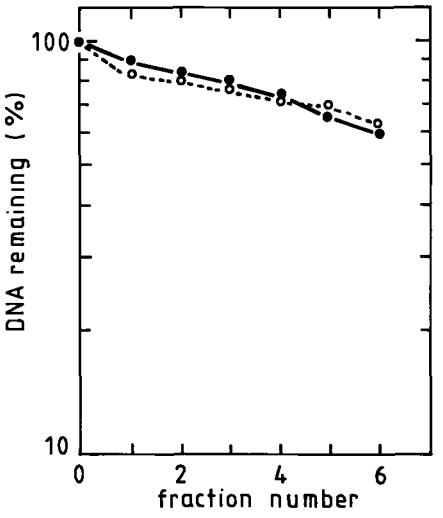

Figure 6.3: Alkaline elution of DNA from non-parenchymal liver cells isolated from a young (●) and an old (o) rat.

6.4 Discussion

Until now there is no general agreement about a possible role of DNA damage in the etiology of aging. In order to address this problem, techniques are required that are sensitive enough to measure low levels of "spontaneous" DNA damage. Furthermore, such techniques should be applicable to freshly isolated cells of various organs and tissues, since it has been shown that changes in DNA repair activity can occur after bringing cells into culture (Mullaart et al., 1988a; Chapter 2). Only recently, reliable techniques became available to measure low levels of DNA damage in freshly isolated non-radioactively labeled cells (Vijg and Uitterlinden, 1987).

In the present study we have used the sensitive alkaline elution assay to measure the amount of spontaneous DNA damage present in liver cells of young and old rats. The use of selective isolation procedures made it possible to separately determine DNA damage in parenchymal and non-parenchymal cells

from the same liver. The results obtained from analysis on 3 young and 3 old rats indicate that upon aging there is an increase in the number of sites detected by alkaline elution, but only in the parenchymal liver cells. The age-related increase is about 700 sites per cell; relative to the level in young rats the difference amounted to almost 80 %. In reality, however, the relative increase probably is higher than this figure; since the zero-level of the method is unknown and cannot be assessed because DNA devoid of any SSB or alkali-labile site is not obtainable, part of the sites detected may be - and very likely are - induced during isolation and testing. The conclusion appears warranted, therefore, that the "physiological" level of SSB and/or alkali-labile sites in DNA of parenchymal cells is at least twice as high in old as in young rats.

The age-related difference of around 700 sites per cell in parenchymal liver cell DNA is considerably smaller (about 30 times) than the increase reported by Ono et al. (1976) and by Su et al. (1984). Using the less sensitive alkaline sucrose gradient method, these authors found about $2\text{-}3x10^4$ alkali-labile sites per cell more in liver cells from old mice as compared to the same cell type from young mice. It should be noted that the level in young animals observed by Ono et al. and by Su et al. was also much higher than in our experiments, viz. about 50,000 and 12,000 per cell, respectively. In consequence, the relative increase of roughly 60-150 % is in better agreement with our results. The discrepancy in the level of alkali-labile sites found in young rats, might be due to species differences or could be an artefact of the cell isolation procedure or the DNA damage detection methods.

Thusfar, we have no information about the mechanisms responsible for the generation of the sites detected. Data from Lindahl and Nydberg (1972) indicate that at a body temperature of 37° C, per cell 10,000 spontaneous depurinations are expected to occur per day. Furthermore, it has been suggested that active oxygen species, generated in vivo as a consequence of normal metabolism and detoxification processes, are important inducers of DNA damage during aging (Cerutti, 1985). Both depurination sites and several forms of oxygen-induced

base damages are alkali-sensitive and will thus be detected in the alkaline elution assay. Most of these spontaneous DNA damages will be repaired rapidly. However, incomplete repair or an age-related decline in the repair capacity might cause an age-related accumulation of DNA damage. We observed no age-related differences in repair capacity in rat skin cells after irradiation with ultraviolet light (Mullaart et al., 1989a; Chapter 3) and only a small (18%) age-related decrease in rat liver cells after treatment with AAF (Mullaart et al., 1989b; Chapter 4). In addition, Ono and Okada (1978) found no difference in repair of damage induced by gamma-irradiation in liver cells from young and old mice. With respect to the age-related increase in SSB and/or alkali-labile sites an alternative possibility, that cannot be ruled out, is that DNA repair itself induces the breaks. During DNA repair, breaks in the DNA are continuously generated and ligated.

Whatever the source may be of the age-related accumulation of the detectable sites in the DNA of rat parenchymal liver cells, this phenomenon was not observed in the non-parenchymal cell fraction isolated from the same livers. In contrast to parenchymal cells, which are (reverting) post-mitotic cells with a very low mitotic frequency, the non-parenchymal liver cells predominantly consist of actively dividing cells (Knook, 1980). Therefore, it is tempting to speculate that an age-related accumulation of DNA damage is restricted to post-mitotic cells, as has been previously suggested by Gensler and Bernstein (1981). This could be due to the presence in these cells of a less active repair system. Several authors found lower levels of DNA repair activities in differentiated post-mitotic cells when compared to actively diving cells (Hahn et al., 1971; Wheeler and Wierowsky, 1983; Vijg et al., 1986a). In actively dividing cells damage has to be removed in order not to interfere with DNA replication, while in post-mitotic cells damage can accumulate until it becomes lethal for the cell. Therefore, cell division preferentially occurs in cells with a minimal amount of DNA damage. In addition, since parenchymal liver cells from rats older than 1.5 month are polyploid (Van Bezooijen et al., 1984) and thus contain more gene copies per cell, they may accumulate more DNA damage before it is lethal.

In addition, it is possible that as a consequence of differences in function, parenchymal and non-parenchymal liver cells are exposed to different types and levels of genotoxic agents. This hypothesis is supported by data from our laboratory, concerning the genotoxic effects on liver cells of in vivo treatment of rats with benzo(a)pyrene (BaP). An increase was found in the number of alkali-labile sites in parenchymal cells 5 h after administration of the BaP, whereas no effect of BaP was found in the non-parenchymal cells (Mullaart et al., 1989c; Chapter 5).

In summary, we conclude that there is a definite age-related accumulation of sites detected upon alkaline elution in the post-mitotic rat liver parenchymal cells, which brings the level at least at twice the value of young animals. It is not known what the effect could be of an accumulation of about 700 of such sites per liver parenchymal cell. These breaks might for example be the initiation point for interchromosomal crossovers which could lead to a loss of tandemly duplicated genes (Strehler, 1986), or could interfere with transcription. With respect to this latter possibility it should be noted that only a small fraction of the total DNA represents active genes (about 3 %). Therefore, only a few alkali-labile sites will be present in areas directly relevant to transcription. On the other hand transcriptionally active parts of the genome might be more sensitive to the induction of DNA damage than transcriptionally silent parts (Yu, 1983). Studies aimed at testing the template activity of damaged and undamaged DNA should give an indication of the physiological relevance of DNA damage accumulated with age.

CHAPTER 7

SPONTANEOUSLY PRESENT DNA BREAKS IN THE RAT BRAIN DURING DEVELOPMENT AND AGING [1]

7.1 Introduction

Alterations in the level of DNA breaks in the mammalian brain could be highly relevant in relation to both development and aging. It has been suggested that during development DNA breaks are involved in differentiation, for example, by mediating DNA rearrangements (Johnstone and Williams, 1982; Farzaneh et al., 1982; Farzaneh et al., 1987). DNA rearrangements are clearly involved in B and T lymphocyte differentiation (Tonegawa, 1980) and it is not inconceivable that certain developmental processes in the brain (e.g. neuronal maturation, competition and selection) may have the same mechanistic basis (Kidson and Dambergs, 1982). In this regard, the selective cell death found in developing brain might be related to apoptosis, a form of programmed cell death associated with DNA degradation (Wyllie et al., 1984). Interestingly, data from Subba Rao (1973) indicate high levels of DNase during early stages of brain development, i.e. at the time when rapid cell proliferation occurs. Such an elevated level of DNase might well induce large amounts of DNA breaks.

During life, DNA breaks and other forms of DNA damage are likely to be induced more or less continuously by a large variety of endogenous and exogenous genotoxic agents (e.g. oxygen radicals, dietary carcinogens). Such DNA lesions may lead to several adverse health effects, either directly by interfering with transcription or via formation of gene mutations as a consequence of mis-replication and/or misrepair (Vijg and Uitterlinden, 1987; Chapter 1).

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[1] This work has been submitted for publication by Mullaart et al., 1989d.

It is not inconceivable that DNA breaks and other DNA damages accumulate during aging as a result of an imperfect repair or an age-related decline in the DNA repair efficiency (Gensler and Bernstein, 1981: Turturro and Hart, 1984). Indeed, results from our laboratory indicate an age-related increase in the level of DNA damage in post-mitotic rat liver parenchymal cells, but not in the non-parenchymal cells (Mullaart et al., 1988b; Chapter 6). Since the brain contains a large percentage of post-mitotic cells, also this organ could undergo an increase in the level of DNA breaks with age. Moreover, owing to the high oxygen consumption in the brain (about 20 % of total body oxygen consumption; Iverson, 1979), high levels of oxygen radicals might be present, which can be expected to induce substantial amounts of DNA breaks or alkali-labile lesions. An additional source of free radicals in the brain is the consumption of oxygen by amino-acid hydrolases and amino oxidase (Hothersall et al., 1982).

Due to a lack of sensitive techniques, thusfar only limited information has been obtained on the levels of naturally occurring DNA lesions in the brain during aging. Ono et al. (1976) and Su et al. (1984), who used the alkaline sucrose gradient centrifugation found no age-related difference in the level of breaks in mouse brain nuclei. However, since the detection limit of this assay is about 10,000 breaks/cell, changes smaller than that could not be ruled out by these authors.

Here we present quantitative data on the occurrence of DNA breaks and alkali-labile lesions in rat brain during early stages of development, adulthood and old age. According to the data, which were obtained by using the highly sensitive alkaline elution assay (detection limit: 500 breaks/cell), the level of the lesions studied is low and constant during development, maturation and aging.

7.2 Materials and Methods

7.2.1 Animals

The animals used were female Wistar-derived WAG/Rij rats and female Brown Norway (BN/BiRij) rats. They were kept under "clean conventional" conditions and fed a pelleted standard rodent diet (AM II, Hope Farms) (Van Zwieten, 1984). Both food and tap water were available ad libitum. Embryos from 6-month old WAG/Rij rats were used on the 12th, 15th and 19th day of gestation (days post-coital (dpc) 12, 15 and 19), counting the first day after overnight conception as day 0 of gestation. The birth of the rats was at day 21 of gestation.

7.2.2 Preparation of tissue suspensions

After decapitation of the mother (6-month old WAG/Rij rat), the cerebral cortex of the rat embryos were rapidly collected (within 20 min) and carefully cut out under a stereo microscope using an ophthalmological iris forceps and scissors. Cerebral cortex and cerebellum from young (24 days and 6 months) and old (36 months) WAG/Rij rats were removed directly after decapitation. All tissues were placed on ice directly after dissection. The tissue (100-200 mg) was cut into small pieces and washed twice with 10 ml PBS/EDTA (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 2.6 mM KCl and 5 mM EDTA, pH 6.7). A suspension was made by gently pipetting up and down the tissue fragments through a pasteur pipet with a 1-mm diameter opening. After filtering through a cotton gauze and centrifugation (10 min at 400 g), the pellet was resuspended in about 10 ml PBS/EDTA. This isolation method resulted in a suspension of nuclei and some disrupted cells. The same procedure was used for the isolation of nuclei from the cerebral cortex of young (6 month) and old (36 month) BN/BiRij rats and for the isolation of liver nuclei to be used as positive controls.

7.2.3 Determination of the number of DNA breaks

The amount of DNA breaks was determined by using the alkaline elution assay as described previously (Mullaart et al., 1989c; Chapter 5), but with some modifications. About $1x10^6$ nuclei, suspended in 0.5 ml PBS/EDTA, were added to 0.5 ml sarkosyl lysis buffer (0.2 % sarkosyl, 2 M NaCl and 0.02 M EDTA, pH 10.0) layered onto a 25-mm diameter polycarbonate membrane filter (Nuclepore; pore size 5 μm). The lysis buffer was allowed to pass through the filter by gravity and replaced by 3 ml sarkosyl lysis buffer containing proteinase K (0.5 mg/ml). After a ½ h-lysis period at 20°C the filter was drained by gravity and the DNA on the filter was washed twice with 5 ml 0.02 M EDTA, pH 10. The DNA was finally eluted through the filter with 0.06 M NaOH, 0.02 M EDTA, pH 12.6 (calculated) at 30 μl/min. Six 4.5-ml fractions were collected. After the elution, the filter with the residual DNA was transferred to a glass vial and, after addition of 4.5 ml elution buffer, irradiated with 100 Gy of ^{60}Co-gamma-rays (Gamma-cell 100, Atomic Energy of Canada) in order to release the DNA. The 6 eluate fractions and the irradiated membrane fraction were neutralized with 0.8 ml 4 M NaCl, 0.6 M NaOH, 1.0 M NaH_2PO_4, which further contained Hoechst 33258 dye (0.5 mg/l). The fluorescence of the Hoechst/DNA complex was measured at 430 nm (filters 3-73, 4-76 and 5-58) by excitation at 370 nm (filters 7-54 and 7-60) in a Pye Unicam LC-FL detector. The elution results were plotted as the log percentage of DNA remaining on the filter as a function of the fraction number. Mean slopes of the linear initial part of three independent elution curves from each sample were used to calculate the number of DNA breaks. To calibrate the alkaline elution assay, nuclei were irradiated (in PBS in Eppendorf vials) at 0°C in the ^{60}Co-gamma-source at a dose rate of 8 Gy/min, for different time periods.

7.2.4 Postmortem stability of brain DNA

The postmortem stability of brain DNA from young and old rats was determined at 0 and 37°C, as follows. Freshly removed rat cerebral cortex was sealed in a plastic bag and placed in a waterbath of 37°C for 3 h or kept at 0°C for the same period. After this period the number of breaks was determined as described above.

7.3 Results

To study the level of breaks present in brain DNA, a highly sensitive alkaline elution assay was used. With this assay, single-strand breaks (SSB) already present in the DNA are measured, together with certain other lesions that are converted into SSB as a consequence of the exposure to alkali during the assay (alkali-labile sites) (Kohn et al., 1978).

Attempts to isolate intact neurons by incubating brain tissue with trypsin as described by Farooq and Norton (1978), were not successful since the DNA of these cells was highly degraded, probably as a consequence of the lengthy procedure and the incubation at 37°C (results not shown). Isolated nuclei, however, contained high molecular weight DNA as determined by alkaline elution (see below). Microscopic examination of the nuclei after staining with crystal violet indicated that, according to the criteria of Kato and Kurokawa (1967), about 60-70 % were of neuronal origin (results not shown).

In order to quantitatively interpret the elution curves, the assay was calibrated by introducing known amounts of SSB in the DNA of brain nuclei by irradiation with different doses of gamma-radiation. Representative elution curves obtained with DNA from unirradiated, 2 Gy or 4 Gy gamma-irradiated nuclei isolated from the cortex of a 6-month old rat, are shown in Fig. 7.1. The slope of these curves represent the rate of elution of the DNA through the membrane filter, which is a measure of the number of SSB and alkali-labile sites. It is clear that

irradiated brain nuclei contain more DNA breaks, as indicated by a steeper slope, than unirradiated nuclei (Fig. 7.1).

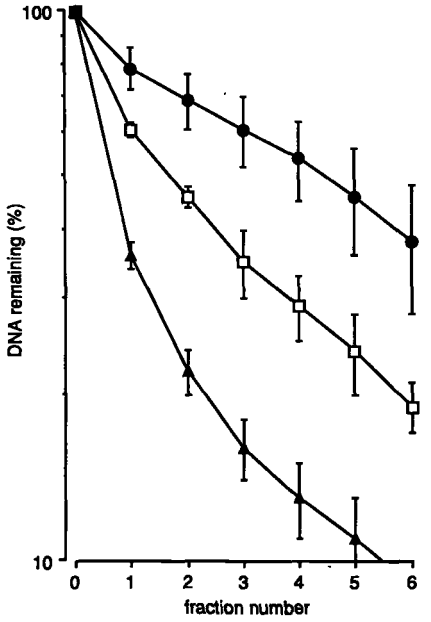

Figure 7.1: Alkaline elution curves of DNA from nuclei after irradiation with 0 (●), 2 (□) or 4 (▲) Gy gamma rays.

When the initial slopes of these alkaline elution curves were plotted against the irradiation dose, a linear dependency was found for the induction of SSB (Fig. 7.2). This calibration curve was used to calculate the number of breaks

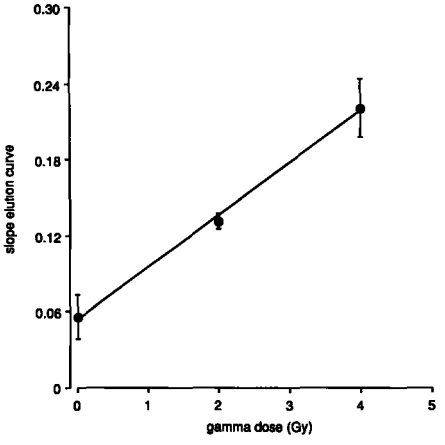

Figure 7.2: Dose response of the induction of breaks by gamma-rays. The number of breaks is expressed as the average initial slope of the elution curve (% DNA remaining per fraction) of three independent determinations. Bars represent the estimated error (SD).

present in the experimental brain DNA samples, on basis of the assumption that 1 Gy gamma-irradiation induces 0.25 SSB/10^9 mw of DNA (corresponding to 1,000 SSB per cell, assuming a total mw of DNA of $4x10^{12}$) (Van der Schans et al., 1982). By using this calibration curve it can be calculated that unirradiated rat brain DNA contains about 0.3 breaks/10^9 mw of DNA.

Typical elution curves obtained with nuclei isolated from cerebral cortex of rat embryos at 12, 15 and 19 days after gestation, and of 24-day, 6- month and 36-month old WAG/Rij rats, are shown in Fig. 7.3. Recently, we demonstrated

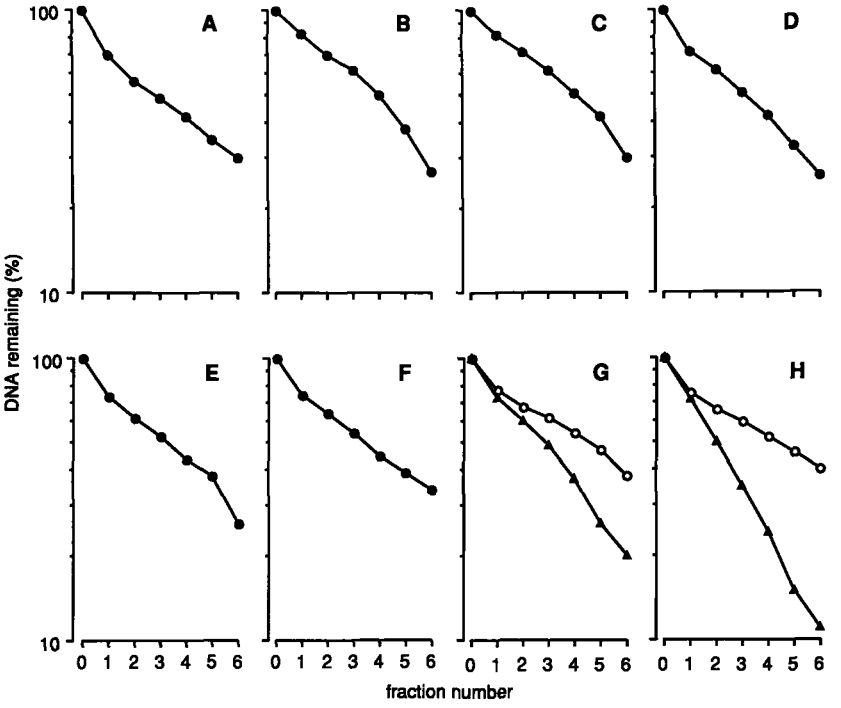

Figure 7.3: Representative alkaline elution curves of DNA from brain nuclei isolated from cerebral cortex of rat embryos at the 12th (A), 15th (B) and 19th (C) day of gestation and of 24-day (D), 6-month (E) and 36-month (F) old WAG/Rij rats. and from liver (▲) and cortex (o) of-6 month (G) and 36-month (H) old BN/BiRij rats.

an age-related increase in the level of DNA damage in isolated BN/BiRij rat liver parenchymal cells (Mullaart et al., 1988b; Chapter 6). Therefore, determinations on DNA from liver and cortex of a young and an old BN/BiRij rat were included in the present experiments. The results shown in Fig. 7.3 make it clear that the level of breaks in brain nuclei remains at the same low level during development, adulthood and aging, as indicated by the almost identical elution curves. In contrast, the results obtained with liver nuclei, isolated according to the same protocol as brain nuclei, clearly indicate an age-related increase in the slope of the elution curve (compare Fig. 7.3G with 7.3H). This essentially confirmed our earlier results on isolated liver cells. No age-related difference in the elution slope was observed in nuclei isolated from the cerebral cortex of the same animals (Fig. 7.3G + 7.3H); these slopes did not differ from those obtained with WAG/Rij rats.

Table 7.1 DNA BREAKS IN CORTEX, CEREBELLUM AND LIVER OF RATS DURING DEVELOPMENT AND AGING.

Rat strain	Organ	Age	Number of breaks per 10^9 mw of DNA
Wag/Rij	cortex	12 dpc[1]	0.372 ± 0.091[2]
		15 dpc	0.396 ± 0.098
		19 dpc	0.317 ± 0.128
		24 days	0.354 ± 0.067
		6 months	0.288 ± 0.054
		36 months	0.293 ± 0.117
	cerebellum	6 months	0.305 ± 0.109
		36 months	0.323 ± 0.097
BN/BiRij	Liver	6 months	0.520 ± 0.136
		36 months	0.991 ± 0.195*
	cortex	6 months	0.273 ± 0.097
		36 months	0.330 ± 0.151

[1] dpc = days post-coital
[2] Mean ± estimated error (SD) of three different animals.
* $P < 0.05$, using Student's t-test

The results of measurements of the level of DNA breaks in brain nuclei of different rats, as compared to the situation for liver nuclei, are summarized in Table 7.1. A constant low level of 0.3 breaks/10^9 mw of DNA (corresponding with about 1,200 breaks per cell) was found in nuclei isolated from the cerebral cortex throughout pre- and post-natal life and aging, while in liver nuclei isolated from BN/BiRij rats an age-related increase of about 0.5 breaks/10^9 mw of DNA (about 2,000 breaks per cell) was found. This difference between liver nuclei from young and old rats is statistically significant ($P < 0.05$, as tested by Student's t-test). Also in cerebellum no difference in DNA break level was observed between 6- and 36-month old rats (Table 7.1).

During our experiments it was observed that the level of SSB in rat brain was increasing with the postmortem interval. This phenomenon was studied in some more detail by keeping brain material at 37°C for 3 h postmortem, next to control samples kept at 0°C for the same period. The results obtained (Table 7.2) clearly indicate that breaks were rapidly induced in the cerebral cortex of

TABLE 7.2 AGE-DEPENDENCY OF THE INDUCTION OF DNA BREAKS IN CEREBRAL CORTEX DURING 3 H POSTMORTEM INCUBATION.

Age (months)	Incubation temperature (°C)	Number of breaks induced/10^9 mw of DNA
6	0	0.09 ± 0.02 [1]
	37	0.50 ± 0.18
36	37	0.61 ± 0.24

[1] *Mean ± estimated error (SD) of three different animals.*

young rats when kept at 37°C, while almost no breaks were induced at 0°C. In view of the possibility that age-related neuronal degradation presensitizes the DNA with respect to the postmortem induction of breaks, we compared the level of breaks induced in brains from young and old rats during a postmortem incubation of 3 h at 37°C. However, about the same number of DNA breaks was found to be induced (Table 7.2).

7.4 Discussion

In the present study we have applied the highly sensitive alkaline elution assay to monitor the level of DNA breaks present in rat brain during prenatal development, adulthood and aging. The results obtained indicate a constant low level of breaks (1,200 per cell) in rat brain nuclei during pre- and postnatal life, which suggests no important role for DNA breaks in brain development. Such a role has often been suggested on the basis of existing evidence that differentiation processes are associated with the appearance of several thousands of transiently present DNA breaks per cell. During differentiation of chick myoblasts about 1,000 breaks per cell were found to be induced (Farzaneh et al., 1982), while resting mouse lymphocytes were reported to contain 3,200 breaks per cell more than mitogen stimulated cells (Greer and Kaplan, 1983). However, until now no information was available about the level of naturally occurring DNA breaks or other DNA damages at different stages of brain development.

The alkaline elution assay used in this study is sensitive enough to detect differences in the order of 500 breaks per cell. Theoretically it is possible that changes in the level of breaks at far lower levels do occur but are not detectable by the method used in this study. It should be realized that the zero-level of the assay is unknown and cannot be assessed because DNA devoid of any SSB or alkali-labile sites can not be obtained. Part of the lesions detected are very likely induced during isolation and testing and consequently the "physiological" level of SSB and/or alkali-labile sites will be lower. It should also be noted that

the results on differentiation-associated alterations in the break levels reported earlier (Johnstone and Williams, 1982; Farzaneh et al., 1982) were obtained by using the nucleoid sedimentation assay. Recent results from our laboratory (Boerrigter et al., 1989a) and from Jostes et al. (1989) suggested that the results obtained by this method do not accurately reflect DNA breaks; other factors, such as DNA-supercoiling might also influence the sedimentation rate of the nuclei.

Besides a possible role in differentiation processes it has been suggested that DNA breaks and other forms of DNA damage accumulate during aging, for instance as a result of an imperfect repair or an age-related decline in DNA repair efficiency (Gensler and Bernstein, 1981; Turturro and Hart, 1984). Our present results indicate that there are no age-related changes in the number of naturally occurring DNA breaks in rat brain nuclei. It should be realized, however, that also in this case it is theoretically possible that there is a very small age-related increase in the level of DNA damage (< 500 breaks per cell), which is not detected by the alkaline elution assay.

In contrast to the situation in brain nuclei, experiments with rat liver nuclei, indicate an age-related increase of about 0.5 breaks/10^9 mw of DNA (about 2,000 breaks per cell). In our earlier experiments using "enzymatically" isolated rat liver parenchymal cells, an age-related increase of about 700 breaks per cells was found (Mullaart et al., 1988b). The fact that the age-related difference found here for liver nuclei (Table 7.1) is larger, might be caused by the isolation procedure used. The "mechanical" isolation method resulted in a higher level of background breaks in liver than the "enzymatic" isolation method; 2,300 and 900 sites, respectively (Table 7.1, Mullaart et al., 1988b). This implicates that the relative increase observed in the present study is about 90 %, which is in good agreement with the value obtained earlier (80%) (Mullaart et al., 1988b). Interestingly, the relative age-related increase found in mouse liver DNA by Ono et al. (1976) and Su et al. (1984), was in the same range, while the absolute age-related increase was much larger than the values obtained in this present

study, namely about 20,000-30,000 breaks per cell. This might be explained by the fact that DNA from old animals is more sensitive to degradation during isolation than DNA from young animals. However, our data on the postmortem induction of DNA breaks did not reveal an age-related difference in DNA stability (Table 7.2).

In summary, we conclude that there are no changes in the level of breaks in rat brain nuclei during prenatal development and aging. Control experiments indicate a twofold higher level of breaks in rat liver nuclei from 36-month old rats as compared with 6-month old rats. The fact that this increase was found in liver and not in brain suggests that an accumulation of DNA breaks is not a general aging phenomena. As already suggested, such DNA breaks might well be induced by radicals generated during metabolization and detoxification in the liver of genotoxic agents present in food (Mullaart et al., 1989c; Chapter 5). Taken together, our results indicate that DNA breaks in rat brain are not of fundamental importance during prenatal development and aging.

CHAPTER 8

INCREASED LEVELS OF DNA BREAKS IN CEREBRAL CORTEX OF ALZHEIMER'S DISEASE PATIENTS [1]

8.1 Introduction

Alzheimer's disease (AD), the most common cause of dementia, is generally characterized by brain atrophy caused by neuronal shrinkage and/or death, neuritic plaques and neurofibrillary tangles (Terry, 1983; Swaab et al., 1986). It has been suggested that the degradation and death of neurons in AD brains is caused by an accumulation of DNA damage, as a consequence of a general DNA repair defect (Robbins, 1983). This hypothesis was derived from the observation that non-neuronal cells such as fibroblasts and lymphocytes isolated from AD patients are more sensitive to gamma rays and certain alkylating agents as compared to cells of the same types derived from normal controls; no defect was observed in the survival of such cells after UV-irradiation (Robbins et al., 1983; Robbins et al., 1985; Scudiero et al., 1986). These observations suggest that AD cells are less able to remove lesions induced by gamma rays and alkylating agents, such as single-strand breaks (SSB) and various small base damages, which are normally removed via short-patch repair (Regan and Setlow, 1974) and many of which are converted into breaks in alkali. Indeed, both Li and Kaminskas (1985) and Robison et al. (1987) reported a decreased capacity to remove alkali-labile sites induced by alkylating agents in fibroblasts from AD patients, when compared to cells from control individuals. However, Kinsella et al. (1988a; 1988b), using the same fibroblast lines, did not observe such a difference. Preliminary results from our own laboratory also indicate proficient repair of alkylating agent-induced alkali-

[1] Parts of this work have been submitted for publication by Mullaart et al., 1989c.

labile sites in fibroblast cell lines from familiar AD patients.

The most direct way of testing the hypothesis that AD is associated with the occurrence of excessive amounts of DNA damage in the brain, is by assaying the amount of DNA breaks in neuronal tissue of AD patients and controls. Indications for an increased level of spontaneous DNA breakage in the brains of AD patients are the observed alterations in the extent of DNA supercoiling (Bachelard et al., 1986) and chromatin structure (Lewis et al., 1981; Crapper McLachlan et al., 1984) in the cortex. Such changes, which might well be due to an increased level of DNA breaks, are important since they might be the underlying cause of the reduced level of total poly(A)+ RNA (Sajdel-Sulkowska and Marotta, 1984) and the loss of a number of messenger RNAs (Crapper McLachlan et al., 1988) observed in the cortex of AD patients. It was therefore considered opportune to establish the level of spontaneous DNA damage in the brains of AD patients in a direct way.

In the present study the highly sensitive alkaline elution assay was used to quantitate the amount of spontaneous DNA breaks in brain samples from AD patients and controls. The results presented here indicate an at least twofold increase in the level of DNA breaks in cortex of AD patients as compared to control subjects. These results are the first direct evidence that accumulation of DNA damage, possibly as a consequence of defective repair, could play a causal role in AD.

8.2 Materials and Methods

8.2.1 Brain material

Human brain material was obtained from rapid autopsies (1.5 - 6 h postmortem delay) and diagnoses were made on the basis of clinical records and neuro-pathological confirmations (Table 8.1) (neuropathological protocol by F.C. Stam, W. Kamphorst, Free University of Amsterdam, the Netherlands

and D. Troost, Academical Medical Center, Amsterdam, the Netherlands). The AD group showed extensive neocortical and hippocampal neuropathological changes (senile plaques and tangles) typical for AD. The areas examined are cortical areas: 4, 10, 17-18, 20, 38 and 42, corpus striatum, gyrus brevus, hippocampus, mesencephalon including substantia nigra, medulla oblongata, thalamus, locus coeruleus and cerebellum. The control brains displayed no neuropathological abnormalities.

Rat cerebral cortex was used from 6-month old female inbred Wistar derived WAG/Rij rats.

8.2.2 Preparation of tissue suspensions

Cerebral cortex was cut into small pieces and washed twice with 10 ml PBS/EDTA (8.1 mM Na_2HPO_4, 15 mM KH_2PO_4, 0.14 M NaCl, 2.6 mM KCl and 5 mM EDTA pH 6.7). A suspension was made by gently pipetting up and down, using a pipet with a 1-mm diameter opening. The suspension was filtered through a cotton gauze and centrifuged (10 min at 1,300 rpm), after which the pellet was resuspended in about 10 ml PBS/EDTA. This isolation method resulted in a suspension of nuclei, 70 % of which could be microscopically identified after staining with crystal violet as neuronal nuclei (Kato and Kurukawa, 1967).

8.2.3 Determination of the number of DNA breaks

The levels of DNA breaks in these nuclei isolated from cortex samples were determined with the alkaline elution assay as described earlier in Chapter 5 and 7. In addition, the amount of breaks in cortex samples from AD patients was determined by means of alkaline sucrose gradient centrifugation, according to a modification of the method originally described by Vijg et al. (1986b). This modification, which involves the use of a mouse monoclonal antibody

against single-stranded DNA instead of an antibody against N-acetoxy-2-acetylaminofluorene-modified DNA, has been described in Chapter 2.

8.3 Results

In principle the alkaline elution method can be used to study various forms of DNA lesions in autopsy material. The breaks detected using this assay are either real SSB already present in the DNA or are derived from DNA lesions, which are turned into breaks during alkali-treatment. In order to estimate the number of DNA breaks present in the brain samples, a set of calibration graphs was prepared by performing elutions with gamma-irradiated human lymphocytes (range 0 - 15 Gy) (Fig. 8.1). The slope of the

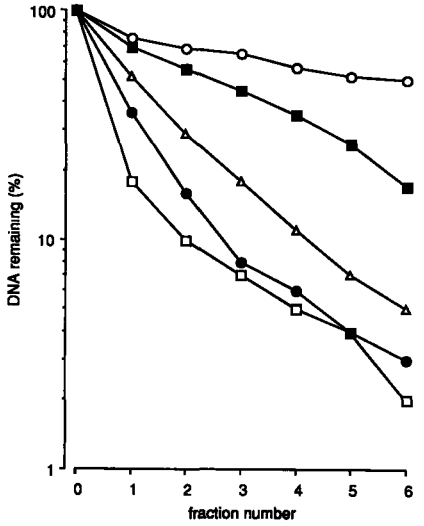

Figure 8.1: Alkaline elution curves of DNA from human lymphocytes after irradiation with 0 (o), 2 (■), 5 (Δ), 10 (●) or 15 (□) Gy gamma-rays.

elution curve represents the rate of elution through the filter, which depends on the number of breaks in the DNA. The experimental curves were fitted to these graphs, the number of lesions was expressed in Gy-equivalents and converted into breaks on basis of the assumption that 1 Gy corresponds to 1,000 breaks per nucleus (Van der Schans et al., 1982). Beyond 10,000 breaks

per nucleus (about 10 Gy) no further discrimination is possible (see Fig. 8.1). Therefore, in brain samples yielding elution curves identical to, or steeper than the calibration curve of cells irradiated with 10 Gy gamma-rays, the level of breaks could not be determined accurately anymore; those samples were considered to contain 10,000 or more breaks per cell.

We first assessed the influence of the postmortem time on the number of breaks detected in human brain samples (Fig. 8.2). When comparing the

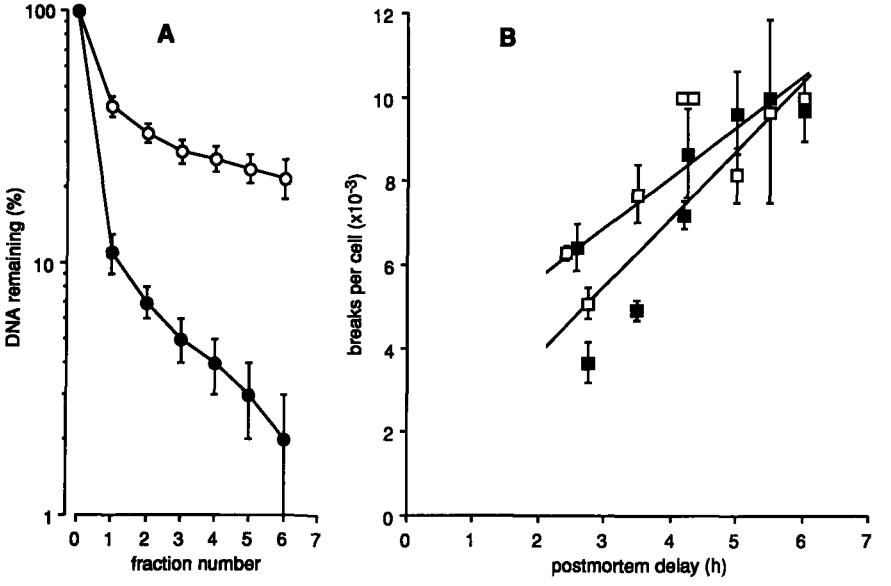

Figure 8.2: Effect of postmortem delay on the number of DNA breaks in human brain samples. (A) Alkaline elution curves of DNA from human occipital cortex with a postmortem delay of 2.5 (○) and 6 h (●). (B) Correlation between the amount of breaks detected in human occipital cortex (□) or frontal cortex (■) and the postmortem delay. Each point is the mean of 3 different elutions with the same brain sample; bars represent the estimated error. Slope of the curve for occipital cortex is 1,188 breaks/nucleus/h, $r = 0.904$; $P < 0.05$, and for frontal cortex 1,589 breaks/nucleus/h, $r = 0.84$; $P < 0.05$).

elution curves of nuclei isolated from brain samples with postmortem delays of 2.5 and 6 h, respectively (Fig. 8.2A), it became clear that the 6-h postmortem sample contains more DNA breaks, as indicated by a steeper slope, than the 2.5-h postmortem sample. The level of breaks in these brain samples could be estimated by comparison with elution curves of gamma-irradiated cell samples (Fig. 8.1). When the level of DNA breaks in occipital and frontal cortex of the 8 human control donors was plotted against the postmortem delay, a fairly linear increase was observed (Fig. 8.2B).

To investigate whether the postmortem induction of DNA breaks is characteristic for human brain sample or is a general phenomenon, we determined the effect of postmortem delay on the level of breaks in rat brain. To mimic the slow temperature drop of the large human brains, freshly removed rat brains were sealed in plastic bags and placed in a container with 30 l of water at 37°C, which was kept at room temperature to cool down as described by Spokes and Koch (1978). After different time intervals, nuclei were isolated from the cortex and used in the alkaline elution assay. Figure 8.3 shows

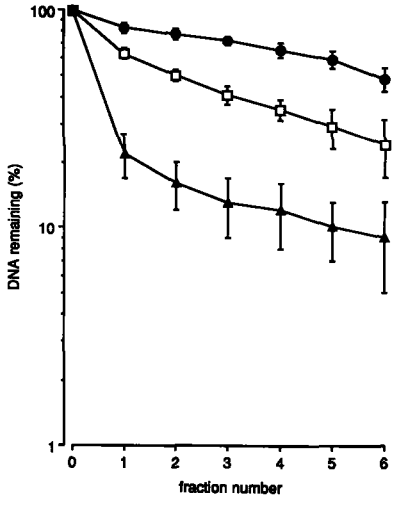

Figure 8.3: Effect of postmortem delay on the number of DNA breaks in rat brain samples. Alkaline elution curves of DNA from cerebral cortex of three different rats with postmortem delays of 0 (●), 3 (□) and 24 h (▲), respectively. Each curve is the mean of 3 different elutions performed on the same rat brain sample (± SD).

typical elution curves of DNA from rat cortex with a postmortem interval of 0, 3 and 24 hrs. It is clear that a prolonged postmortem delay resulted in an accelerated elution rate, indicating an increased level of DNA breaks. When plotted against postmortem interval, the level of DNA damage in rat cerebral cortex showed a linear increase (Fig. 8.4). However, the induction of breaks in rat brains seems to occur less rapid than in human brains (compare Figs. 8.4 with 8.2B).

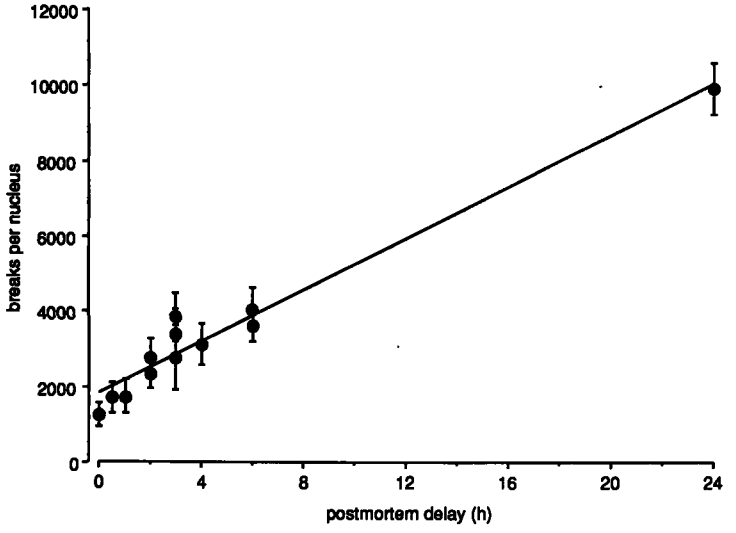

Figure 8.4: Correlation between the amount of breaks per nucleus in rat brain samples and the postmortem delay.

Then we determined the level of DNA breaks in brain samples from AD patients. Typical elution curves obtained with DNA from the cerebral cortex of a control individual and of an AD patient both with short postmortem intervals (2.75 h) are shown in Fig. 8.5. The slopes are clearly different, the steeper slope belonging to DNA isolated from the AD patient. When comparing the level of DNA breaks in cerebral cortex of 11 AD patients with that of 3 control donors at short postmortem delays (i.e. $\leqslant 4$ h) a twofold higher level was observed in AD patients (Table 8.1).

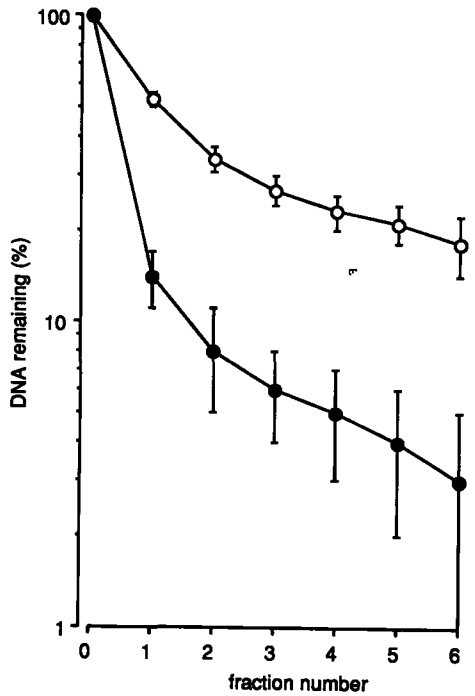

Figure 8.5: Typical elution curves of DNA obtained from the occipital cortex of an AD patient (●) and a control individual (o).

In order to compare all human brain samples obtained, the linear relationship between postmortem delay and the level of DNA breaks (Fig. 8.2B) was used to normalize all values to a postmortem delay of 2.7 h (mean postmortem delay of the AD group). The results confirmed the difference

Table 8.1 HUMAN BRAIN SAMPLES

Donor	Age (years)	Sex	Brain weight (g)	PM delay (h)[a]	PH	Cause of death	Damages per nucleus[b] ($x10^{-3}$) uncorrected Occipital	uncorrected Frontal	corrected[c] Occipital	corrected[c] Frontal
A) controls[d]										
87-290.0	51	f	1190	2.75	6.50	Broncho alveolar carcinoma; no metastases in brain.	5.1 ± 0.4	3.7 ± 0.5	5.0 ± 0.4	3.6 ± 0.5
87-340.4	76	m	1245	2.5	6.89	Lung carcinoma; no metastases in brain.	6.3 ± 0.2	6.4 ± 0.6	6.5 ± 0.2	6.7 ± 0.6
88-9.3	69	f	nd	6.0	6.10	Septic shock in Non-Hodgkins Lymphoma following treatment with cytostatics.	> 10	9.7 ± 0.7	> 6.0	4.2 ± 1.4
88-29.4	60	m	1350	5.0	6.45	Septic shock following aorta valve implantation.	8.1 ± 0.7	9.6 ± 0.9	5.4 ± 0.9	5.8 ± 1.3
88-194.3	65	m	1310	4.25	6.18	Heart failure.	> 10	8.7 ± 1.0	> 8.1	6.1 ± 1.2
88-231.2	73	m	1410	3.50	6.93	Post-operative heart failure.	7.7 ± 0.7	4.9 ± 0.2	6.7 ± 0.8	3.6 ± 0.4
88-328.1	66	f	1100	5.5	6.24	Post-operative heart failure.	9.7 ± 2.2	> 10	6.3 ± 2.3	> 5.4
88-366.0	71	f	1240	4.25	6.28	Sepsis and cardiogenic shock.	> 10	7.2 ± 0.3	> 8.1	4.7 ± 0.7
mean	66		1263	4.22	6.45				6.5	5.0
SEM	(3)		(42)	(0.44)	(0.11)				(0.4)	(0.4)
B) AD patients[d]										
87-318	88	f	1070	2.75	6.93	Pneumonia.	> 10	> 10	> 9.9	> 9.9
87-329	88	f	960	2.25	6.30	Non-Hodgkins lymphoma, broncho pneumia, heart failure.	> 10	> 10	> 10.5	> 10.7
88-082	81	f	990	2.50	6.32	Heart failure.	> 10	8.5 ± 0.9	> 10.2	8.8 ± 0.9
88-090	70	m	865	2.50	6.14	Subdural hematome.	9.4 ± 3.7	6.0 ± 0.6	9.7 ± 3.7	6.3 ± 0.6
88-109	85	f	1020	1.50	6.32	Intestinal bleeding.	> 10	9.3 ± 0.4	> 11.5	11.3 ± 0.6
88-252	66	m	1250	2.25	6.50	Cachexia and presumable sepsis.	9.3 ± 1.1	7.1 ± 0.7	9.8 ± 1.1	7.9 ± 0.7
88-255	82	f	980	3.50	6.40	Sepsis; Pneumonia.	> 10	> 10	> 9.0	> 8.7
88-272	84	f	1000	3.75	6.42	Septic shock; cachexia; basilaris artery insufficiency	9.9 ± 0.7	> 10	8.7 ± 0.8	> 8.3
88-296	88	f	940	2.5	6.42	Myocard infarct.	> 10	> 10	> 10.2	> 10.3
88-303	79	f	1190	4.0	6.44	Cachexia and internal bleeding as result of chronic myeloid leukemia.	9.9 ± 0.4	9.7 ± 0.2	8.3 ± 0.6	7.6 ± 0.5
88-325	64	f	800	2.5	6.44	Cachexia and dehydratation.	8.6 ± 0.2	8.2 ± 0.2	8.9 ± 0.2	8.5 ± 0.2
mean	80		1006	2.73	6.42				9.7	8.9
SEM	(3)		(39)	(0.22)	(0.06)				(0.3)	(0.4)
C) Parkinson's patients[d]										
87-235	80	f	1180	3.0	6.99	Pneumonia.	5.7 ± 0.3	6.8 ± 0.7	5.3 ± 0.3	6.3 ± 0.7
88-072[e]	92	f	1020	3.5	6.09	Heartfailure.	> 10	8.3 ± 0.8	> 9.0	7.0 ± 0.9

[a] The PM delay (postmortem delay) is defined as the period between death and the moment the brains were placed on ice.
[b] The number of damages per nucleus is given as the mean (± SD) of 3 different elutions performed on the same brain sample.
[c] Amount of damages per nucleus after normalization to 2.7-h postmortem delay.
[d] The diagnosis of the subjects as controls, AD patients or Parkinson disease patients was made as described in the section 8.2.1.
[e] Patient having the neuropathological signs of both Parkinson and AD.

nd = not determined

seen after selection for short postmortem delays: the level of DNA breaks in both occipital and frontal cortex of the AD group was significantly higher than that in the control group ($P < 0.0005$, as tested by Student's t-test; Table 8.1).

During the course of this study, brain samples from 2 patients having Parkinson's disease became available. One of these had the neuropathological features of both Parkinson and AD (88-072). Interestingly, the cortex from the other patient who had Parkinson's disease without dementia (87-235), had low levels of DNA breaks, whereas the DNA of the Parkinson/AD patient DNA contained about twice as many lesions per nucleus (Table 8.1).

Since several AD brain samples contain too much damage to be accurately quantified with the alkaline elution assay, we applied the less sensitive alkaline sucrose gradient centrifugation. Typical sedimentation profiles of two AD samples (88-090 and 88-318) are shown in Fig. 8.6. From these sedimentation profiles it can be calculated that these AD samples contain 20,000 (AD 88-090) or 60,000 (88-318) breaks per nucleus. It should be realized that this

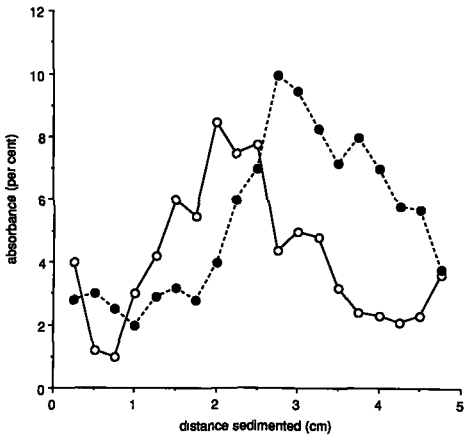

Figure 8.6 Alkaline sucrose gradient sedimentation profiles of DNA from the occipital cortex of two AD patients (● = 88-090 and o= 87-318).

method is less sensitive than the alkaline elution assay. In addition, in case of the alkaline sucrose gradient centrifugation assay extra DNA breaks can

be induced during isolation and testing of the DNA. For instance, control rat brain DNA appeared to contain already 20,000 breaks per cell as measured by sucrose gradient sedimentation (results not shown), while with the alkaline-elution only about 1,200 breaks were found (Fig 8.4).

8.4 Discussion

A highly sensitive alkaline elution assay was used to quantitate the level of "spontaneous" DNA breaks in the cerebral cortex from AD patients and controls. Our data on 11 AD patients and 8 control individuals, corrected for postmortem delay, indicate an at least twofold higher level of DNA breaks in cortex of AD patients as compared to controls. Comparison of the uncorrected number of breaks in AD and controls selected for short postmortem intervals, reveals the same difference.

The possibility that the difference in breakage level between AD and control is related to the difference in mean age (66 ± 3 and 80 ± 3 for controls and AD patients, respectively), is unlikely since the levels of DNA breaks in the two youngest AD patients (64 and 66 years of age) are twice as high as the levels in the two oldest control subjects (73 and 76 years of age). In addition, no age-related difference was observed in the level of DNA-breaks in rat brain (Mullaart et al., 1989d; Chapter 7).

It should be noted that the number of breaks in the cortex DNA of many AD patients, and some controls, was so high that very steep elution curves were obtained, which allowed only the estimation of the minimal levels of DNA breaks ($>10,000$ per nucleus). On the average, this phenomenon very likely has resulted in an underestimation of the amount of lesions present in most of the AD patients relative to controls. The question may arise whether in these samples still lesions in intact DNA were determined, or that strongly degraded DNA was assayed predominantly originating from necrotic cells. Microscopic examination of the brain samples did not favor the latter

explanation, however; the nuclei had the normal appearance. Moreover, when alkaline sucrose gradient centrifugation was applied, a less sensitive method for detection of DNA breaks (Vijg et al., 1986b; Mullaart et al., 1988a, Chapter 2), extensively degraded DNA was not observed; the levels remained below 60,000 breaks per nucleus. This result was confirmed by using field inversion electrophoresis (Carle et al., 1986) experiments, which demonstrated that the DNA of the AD and control samples tested was of high molecular weight (longer than 10^6 basepairs) (results not shown).

Nevertheless, it should be realized that the increased level of DNA damage observed in the cortex of AD patients could still be an effect rather than the cause of the disease. For example, even moderate increased DNA breakage could be a side effect of neuronal degeneration or could result from an extended agonal state. Although this possibility can not be ruled out completely, several points argue against it. In the first place, the level of DNA breaks in AD brains was found to be slightly higher in occipital than in frontal cortex, while cell degeneration and cell death is generally more severe in the frontal cortex (Coleman and Flood, 1987; Stewart et al., 1988). Furthermore, the fact that the cerebral cortex of a Parkinson's patient did not contain elevated levels of DNA damage, might suggests that an increased level of DNA breaks is specific for AD and not a general phenomenon associated with neuronal degeneration. Secondly, the pH of the brain, which is a good indicator for the agonal state of the patients (Hardy et al., 1985), did not differ between AD patients and control individuals (Table 8.1; $P > 0.5$ as tested by Student's t-test).

On the basis of the results presented above we interpret our data as evidence of a disturbed DNA-damage metabolism in AD patients. In this respect it is tempting to seek the cause of this disease in a general defect in a DNA-repair pathway via which brain-specific DNA lesions are removed as proposed by Robbins (1983). However, the elevated level of DNA damage in AD brains could just as well result from a higher rate of damage induction as a

consequence of alterations in cellular metabolism. For example, there is evidence for a higher rate of lipid peroxidation in AD brains (Martins et al., 1986), which might lead to an increase in DNA-damage induction.

CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

As a result of a permanent exposure to low levels of various endogenous and exogenous genotoxic agents, large numbers of lesions are continuously induced in the DNA of cells. When not repaired, such lesions could lead to dysfunction of cells and tissues; they might well be the underlying cause of the age-related reduction of homeostatic capacity and the increased incidence of cancer and other diseases of old age (Gensler and Bernstein, 1981; Vijg, 1987).

The cell has evolved multiple repair systems to remove DNA damage, a process which is essential for the maintenance of the integrity of the genome. However, imperfections - even minor ones - in the DNA-repair activities or in the fidelity of the repair could lead to a gradual accumulation of DNA damage during aging. This process would be accelerated in case of the occurrence of an age-related decline in the repair capacities of cells. Until recently, most studies used cultured cells for the determination of DNA damage and repair in relation to aging. Attempts to determine the level of DNA damages and the amount of repair in various cell types in vivo in relation to age have long been hampered by the absence of sensitive techniques. In addition, there was the lack of knowledge about the types of DNA lesions likely to arise and/or accumulate "spontaneously" during aging in certain cell types and organs.

The aim of this study was to investigate (1) the capacity of rat skin and liver cells in vivo to remove low levels of specific lesions in DNA in relation to age; (2) the possible occurrence of an age-related accumulation of DNA damage, i.e. single-strand breaks (SSB) and alkali-labile sites, in rat liver and brain; and (3) the possibility that Alzheimer's disease is associated with an elevated level of SSB and alkali-labile sites in cerebral cortex.

9.1 Does the capacity to remove specific DNA lesions declines with age?

Until now, most studies on DNA repair have focussed on the analysis of specific steps in the process of DNA-damage removal in cultured mammalian cells after treatment with genotoxic agents in vitro. The relevance of these results for the in vivo situation appeared debatable, however, in view of the fact that repair capabilities of cells in vitro do not necessarily reflect the capacity of the same cells in vivo. Therefore, when methods became available to determine the level of DNA damage and repair in various cell types in vivo (Vijg and Uitterlinden, 1987), it was decided first to pay attention to an in vitro/in vivo comparison of DNA repair activities.

To this end the removal of a well defined model lesion, the UV-induced pyrimidine dimer, in the DNA in rat skin cells was studied, both in cultured cells and in vivo. The results indicated a clear difference in the fractional removal of pyrimidine dimers: cultured keratinocytes removed only 20 % of the dimers within 24 h, whereas in cells of the same type in vivo about 50 % were removed within 3 h (Mullaart et al., 1988a; Chapter 2). Evidently, it appears that rat keratinocytes undergo a strong reduction in their capacity to perform long patch excision repair, when they are transferred into culture. On the other hand, results from Meek et al. (1980) and Vijg et al. (1986) indicate an increase in the amount of UV-induced UDS (a measure for the DNA synthesizing step in the excision repair process) in cultured skin fibroblasts during the first cell divisions after their release from the skin. It seems that when cells are transferred from the resting state in the skin to a state of active proliferation in culture, their capacity to remove pyrimidine dimers is greatly diminished, whereas the ability to perform UDS is enhanced. This might be explained by the fact that UV-induced UDS and dimer removal, although both a consequence of the process of excision repair, are two independent steps. This possible explanation is supported by the observation that the creation of repair patches (repair replication) can occur without

any dimer removal (Vijg et al., 1984). Such alterations in the utilization of certain reactions of DNA repair pathways, as a consequence of alterations in environmental circumstances, should lead to cautiousness in the extrapolation of levels of DNA repair determined in cells in vitro to cells in the in vivo situation.

The difference in the extent of DNA repair between cells in vitro and in vivo (Mullaart et al., 1988a; Chapter 2), was given further attention in our study on the extent of repair of UV lesions in skin cells from young and old rats. The results obtained indicated the absence of an age-related decline or any other change in the removal of low levels of UV-induced pyrimidine dimers in rat epidermal keratinocytes (Mullaart et al., 1989a; Chapter 3). Cells from both young (6 months) and old (36 months) rats were found to remove about 50 % of the dimers within 3 h in vivo, while the 20 % removal over 24 h in vitro appeared not to alter with donor age either (Mullaart et al., 1989a; Chapter 3). Evidently, the capacity of rat skin cells to remove a specific UV-induced DNA lesion does not change with age.

The question arose whether this lack of age-related alterations in DNA-repair activity was specific for the skin or for UV-lesions, or that it reflected a more general situation. The highly sensitive ^{32}P-postlabeling assay (Gupta et al., 1982; Gupta, 1985) was used to determine the rate of induction and disappearance of aminofluorene (AF)-adducts in DNA of rat liver cells after intraperitoneal injection with low doses of 2-acetylaminofluorene (AAF). The data indicated an age-related decrease in the rate of disappearance of AF-adducts from rat liver DNA (Mullaart et al., 1989b; Chapter 4). The extent of this decrease (about 18% between 6- and 36-month old rats), however, appeared too small to be of biological significance.

On the basis of the results obtained with both skin and liver cells of the rat, one would be tempted to conclude that there are no substantial age-related changes in DNA-repair activities. With the exception of a study by Ishikawa and Sakurai (1986), who found a slight reduction of the amount

of UV-induced UDS after high UV-B doses in mouse skin cells, no additional information is available about the age-dependency of the capability to perform DNA repair after UV or AAF treatment in vivo. It should be noted that both pyrimidine dimers and AF-lesions are so-called bulky adducts, which are repaired via "long-patch" repair (Regan and Setlow, 1974). The possibility remains, therefore, that age-related changes exist in one or more of the several other repair systems. However, the capacity of mouse brain (Wheeler and Wierowski, 1983; Ono and Okada, 1978), spleen (Ono and Okada, 1978) and liver cells (Ono and Okada, 1978) to remove gamma-induced single-strand breaks (SSB) from their DNA, appeared not to change with the donor age. In addition, no age-related difference was found in the disappearance of alkali-labile sites induced by gamma-radiation and ethyl nitrosourea in the DNA of human lymphocytes (Boerrigter et al., 1989b). Since SSB and alkali-labile sites are repaired via "short patch" repair (Regan and Setlow, 1974), it can be concluded that there is no age-related decline in this repair pathway either.

On the basis of both our own results and those from others mentioned above (for reviews, see Tice and Setlow, 1985 and Hanawalt, 1987) we conclude that there is no evidence for a substantial age-related decline in DNA repair activities.

9.2 Does DNA damage accumulates with age?

Although the level of DNA repair appears to remain fairly constant during aging, our results on both rat skin and liver cells indicate that part of the DNA damage may be persistent, both in young and old rats. About 40 % of the pyrimidine dimers is still present in epidermal keratinocytes at 48 h after irradiation (Mullaart et al., 1989a; Chapter 3), while about 30 % of the AF-adducts can be detected as late as 21 days after treatment (Mullaart et al., 1989b; Chapter 4). The latter results are in fair agreement with those of Visser and Westra (1981), who found that about 15 % of both AF- and

acetyl-AF-adducts were still present 2 weeks after treatment. This incomplete repair will lead to an accumulation of unrepaired DNA lesions during frequently repeated or continuous exposure to genotoxic agents. Indeed, repeated treatment with N-hydroxy-AAF or prolonged treatment (for several weeks) of rats with AAF led to an elevated level of adducts in rat liver (Beland et al., 1982; Huitfeldt et al., 1987).

The most direct way to test the hypothesis that DNA damage accumulates with age, is to monitor the level of spontaneous DNA damage in cells derived from individuals of different ages. A serious problem in this respect is the lack of precise information on the DNA lesions that might accumulate. Nevertheless, from Chapter 1 (section 1.2.1) it can be derived that SSB, AP-sites and small base damages are forms of DNA damage that are likely to occur frequently in vivo. Potential sources in this respect are body heat and free radicals. Results from Ames et al. (1983) also indicate that, for instance, free radicals generated during oxygen metabolism are a potent source of DNA damage during aging. However, also exogenous environmental carcinogens are able to induce SSB, AP-sites and small base damages via radicals generated during metabolic activation and detoxification. Indeed, it was found that intragastrically administered benzo(a)pyrene (BaP), a carcinogen present in tobacco smoke and many common foods, does not induce bulky DNA adducts in its main target organs, the liver and the intestine, as might have been expected; instead the induction of DNA breaks and alkali-labile sites was observed (Mullaart et al., 1989c; Chapter 5). Data from Lorentzen and Ts'o (1977) and Leadon et al. (1988) indicate that during the metabolic activation of BaP in vitro free radicals are generated, which can induce oxidative DNA damage. Therefore, we suggest that breaks and alkali-labile sites found by us were induced via free radicals generated during the metabolization of BaP in liver and intestine. It is not inconceivable that many other compounds present in our environment are processed in a way that generates free radicals. If this concept is correct, damage induced by free radicals might be a good

candidate to accumulate with age, which is in agreement with the free radical theory of aging (Harman, 1981).

In order to investigate whether these damages, i.e. SSB, AP-sites and small base damages, indeed accumulate during aging the highly sensitive alkaline elution assay was used to monitor DNA damage levels in liver and brain from young and old rats. An increase of about 700 damages per cell (80 % of the background level) was found when we compared liver parenchymal cells from 6- and 36-month old rats (Mullaart et al., 1988b; Chapter 6). This difference was observed only in the DNA of post-mitotic parenchymal rat liver cells, and not in that of the nonparenchymal cells that still had proliferative capacity (Mullaart et al., 1988b; Chapter 6).

The fact that in the liver the age-related increase was observed in parenchymal cells only, is compatible with the hypothesis that post-mitotic cells exhibit more pronounced symptoms of cellular senescence than actively dividing cells (Knook, 1980). This higher vulnerability could be caused by an increased rate of DNA damage accumulation, which, on its turn, might be caused by a lower DNA repair capacity of post-mitotic cells (see for instance Hahn et al., 1974). Furthermore, as described in Chapter 1 (section 1.3.2), frequent cell division may force the cell to remove lesions that would otherwise interfere with DNA replication. In non-dividing cells this immediate need to remove lesions is not present and they therefore might tolerate higher levels of DNA damage.

To investigate whether the age-related accumulation of DNA breaks and/or alkali-labile sites found in liver parenchymal cells is a general phenomenon of post-mitotic cells, the level of spontaneous DNA breaks and alkali-labile sites was determined in the rat brain in relation to age. Since the oxygen consumption in the brain is high, large amounts of "oxygen" radicals will be generated and high levels of oxygen-induced DNA damage might be expected. However, in contrast to the data obtained with post-mitotic liver cells, no age-related increase in the level of SSB and alkali-labile sites was

observed in brain nuclei; the level of sites remained constant from prenatal stages of development until the age of 36 months (Mullaart et al., 1988d; Chapter 7).

The occurrence of an age-related accumulation of alkali-labile sites in parenchymal liver cells, but not in brain cells, might reflect differences in the nature and amount of "spontaneous" DNA damage between these two organs. The liver plays an important role in the metabolization and detoxification of several carcinogens. These carcinogens or the reactive byproducts (e.g. radicals) generated during their metabolization can induce DNA damage, as is suggested by the DNA breaks and alkali-labile sites induced during metabolization of BaP in the liver and intestine (Mullaart et al., 1989c; Chapter 5).

Based on the above, the possibility should be considered that the damage accumulation found in the liver is probably caused by radicals generated during metabolic reactions occurring specifically/or predominantly in the liver. This implicates that a low metabolic activity might sometimes be of advantage for the cell since this would lead to a low level of radicals and decreased rate of DNA damage induction. Indeed, an inverse correlation has been found between the ability of cultured fibroblasts to metabolize 7,12-dimethyl-benz(a)anthracene and the maximum lifespan of the species from which the cell cultures were derived (Schwarz and Moore, 1977; Moore and Schwarz, 1978).

For post-mitotic cells, at the moment nothing is known about the relationship between the number of lesions and their effects on proper cell functioning. In other words, is it possible that an age-related increase of 700 breaks and/or alkali-labile sites as observed in parenchymal lever cells, can lead to adverse effects as has been observed on the organ level (Van Bezooijen, 1986). It should be realized that the alkaline-elution procedure detects only SSB and alkali-labile lesions, and the age-related increase of DNA damage might be higher than the 700 lesions/cell found here (Mullaart et al., 1988b;

Chapter 6). Results from Massie et al. (1972b) indicate that about 90 % of the damage induced by the oxygen radical (H_2O_2) are base damages, many of which might not be alkali-labile.

As mentioned above, non-dividing post-mitotic cells may tolerate a considerable amount of DNA damage and this can lead to an gradual accumulation during aging. Such accumulated DNA lesions might be responsible for an age-related deregulation of gene expression, loss of proper cell functioning and ultimately to a loss of cells (see also Chapter 1, section 1.4). An acceleration of this process could lead to cell death much earlier. In this regard there are a number of diseases suggested to be caused by excess DNA damage, for example, as a consequence of a DNA repair defect. One of such diseases is Alzheimer's disease.

9.3 Alzheimer's disease and DNA damage accumulation: cause or effect?

It has been suggested that Alzheimer's disease (AD) represents an acceleration of normal brain aging (Coleman and Flood, 1987; Dr. D.F. Swaab, personal communication). Several alterations observed in AD patients, such as a neuronal loss and a high number of senile plaques, were also found during normal aging but in a less severe form. Interestingly, analogous to the hypothesized age-related accumulation of DNA damage it has been suggested that also AD is associated with elevated levels of DNA damage, as a consequence of a general DNA-repair defect (Robbins, 1983). This damage accumulation might be the underlying cause of the neuronal degeneration and death observed in AD patients. Attempts to test the activity of DNA-repair systems in non-neuronal cells from AD patients and controls thus far have provided conflicting results (see Mullaart et al., 1989e; Chapter 8).

Alternatively, the hypothesis of Robbins can be directly tested by comparing the level of spontaneously present DNA damage in neuronal tissue of AD patients and controls. The results presented here indicate that DNA from

AD brains contains at least twice as many DNA breaks, as detected by the alkaline elution assay, as compared to control brain samples (Mullaart et al., 1989e; Chapter 8). Although this is in perfect agreement with a defect in some aspect of DNA repair as proposed by Robbins (1983), the possibility that this phenomenon is an effect rather than the cause of the disease can not be completely ruled out. It was demonstrated that the increased DNA breakage in AD brains is not likely to be a side effect of neuronal degradation nor a result from an extended agonal state (Chapter 8). However, the elevated level of DNA damage in AD brains could just as well result from a higher rate of damage induction as a consequence of alterations in cellular metabolism. For example, there is evidence for a higher rate of lipid peroxidation in AD brains (Martins et al., 1986), which might lead to an increase in DNA-damage induction. This would mean that the breaks and/or alkali-labile sites present in the brains of AD patients at a high level are not the primary cause but a secondary effect of the disease.

9.4 Summary and General Conclusions.

In this thesis data are described on DNA damage and repair in relation to aging and Alzheimer's disease. Since Hart and Setlow (1974) first demonstrated that the amount of DNA-repair synthesis in UV-irradiated fibroblasts correlated well with the maximum lifespan of the donor species, numerous studies on DNA repair and aging have been performed. Almost without exception these studies were hampered by the lack of adequate techniques to monitor low, physiological levels of DNA damage and its repair. Only recently sensitive and accurate methods were introduced by some research groups including our own (Vijg and Uitterlinden, 1987), which allow, for the first time, to address the question of whether aging and age-related disorders like AD are correlated with "spontaneously" occurring DNA damages.

From the results described in this thesis a number of conclusions can be drawn:

1. There is a clear difference in the extent of pyrimidine dimer removal between rat skin cells in vitro and in vivo.
2. There is no general age-related decline in the capacity of rat cells to remove certain specific DNA lesions neither in vitro nor in vivo.
3. A substantial part of the damage induced in vivo by treatment with low doses of genotoxic agents persists for long time periods.
4. There is an age-related accumulation of alkali-labile sites in DNA of post-mitotic rat liver cells, but not in that of rat brain nuclei.
5. DNA from cerebral cortex of AD patients contains an elevated level of alkali-labile sites as compared to that of brain samples from controls.

SAMENVATTING

Elk levend wezen staat voortdurend bloot aan lage concentraties endogene en exogene genotoxische agentia. Als gevolg daarvan worden voortdurend grote hoeveelheden DNA-beschadigingen geïnduceerd. Indien deze DNA-schades niet hersteld worden kunnen ze zich ophopen en afhankelijk van de plaats in het DNA aanleiding geven tot een verminderd functioneren van cellen en organen. Er is vaak gesuggereerd dat dit de onderliggende oorzaak zou kunnen zijn van veroudering en verouderings-ziekten zoals kanker, arteriosclerose en de ziekte van Alzheimer.

Gelukkig beschikt de cel over een scala aan enzymsystemen om beschadigingen in het DNA te herstellen. Deze DNA-herstel systemen zijn echter niet perfect; een gedeelte van de schade wordt niet hersteld en kan gedurende lange tijd aanwezig blijven. Dit kan leiden tot een geleidelijke ophoping van DNA-schades met het ouder worden. Een eventuele leeftijdsgeassocieerde achteruitgang in de efficiëntie van DNA-herstelprocessen kan deze accumulatie van schades versnellen.

Er zijn de afgelopen jaren, door verschillende onderzoekers, een groot aantal experimenten uitgevoerd om na te gaan of er inderdaad zo'n ophoping van DNA-schades plaats vindt tijdens het ouder worden. De resultaten van deze experimenten zijn echter niet eenduidig en vaak zelfs tegenstrijdig. De oorzaak hiervan is een gebrek aan gevoelige bepalingsmethoden voor DNA-schades en herstel en het gebrek aan kennis omtrent het type DNA-schade dat kan ontstaan en dat zich eventueel kan ophopen tijdens veroudering.

Het doel van de studie beschreven in dit proefschrift was na te gaan in hoeverre (1) de capaciteit van huid en levercellen om specifieke DNA-beschadigingen te herstellen afneemt gedurende het ouder worden van de rat; (2) DNA-beschadigingen zich ophopen in rattelever en hersencellen tijdens het ouder worden en (3) de ziekte van Alzheimer met een verhoogd niveau van DNA-schade geassocieerd is.

Vrijwel alle studies naar de relatie tussen DNA herstel en de leeftijd waren tot nog toe gericht op de bepaling van specifieke stappen in het herstelproces in gekweekte cellen. Het is echter de vraag in hoeverre de herstelcapaciteit van gekweekte cellen een weerspiegeling is van de situatie in dezelfde cellen in het lichaam (in vivo). Daarom werd in de studie die het onderwerp vormt van dit proefschrift in de eerste plaats onderzocht of er inderdaad een verschil bestaat tussen DNA-herstel activiteiten van cellen in vitro en in vivo. Hiertoe werd de verwijdering van ultraviolet-licht geïnduceerde pyrimidine dimeren gemeten in rattehuidcellen (keratinocyten) in vitro en in vivo. De resultaten laten zien dat er inderdaad een duidelijk verschil is; gekweekte keratinocyten verwijderen slechts 20 % van de geïnduceerde schade gedurende 24 uur, terwijl dezelfde cellen in vivo 50 % van de schade verwijderen binnen 3 uur na bestraling (hoofdstuk 2). Dit betekent dat bij de rat de herstelcapaciteit van keratinocyten drastisch vermindert wanneer deze in kweek gebracht worden. De conclusie is dat men zeer voorzichtig dient te zijn met de extrapolatie van DNA-herstel activiteiten gemeten in vitro, naar de in vivo situatie.

Vervolgens werd nagegaan in hoeverre de capaciteit van huidcellen om pyrimidine dimeren te verwijderen afneemt met de leeftijd van de rat. Dit werd bepaald in keratinocyten van ratten, zowel in vitro als in vivo. De resultaten van deze experimenten laten zien dat er in beide situaties geen leeftijdsgeassocieerde afname is in het percentage herstel; keratinocyten van zowel jonge als oude ratten verwijderen ongeveer 50 % van de dimeren in vivo, terwijl ook de 20 % verwijdering gevonden na 24 uur herstel in vitro niet verder afneemt met het ouder worden (hoofdstuk 3).

Het is echter de vraag of dit ontbreken van een verouderings- geassocieerde afname een algemeen fenomeen is of dat dit alleen geldt voor het herstel van ultraviolet licht geïnduceerde pyrimidine dimeren. Daarom werd de inductie en verdwijning bepaald van DNA-schades geïnduceerd door het carcinogeen 2-acetylaminofluoreen (AAF) in rattelever. In dit geval bleek inderdaad dat

de snelheid waarmee de DNA-schade verdwijnt lanzamer is in oude ratten dan in jonge ratten (hoofdstuk 4). Deze afname was echter zeer gering (18%) en het is onbekend wat de biologische betekenis hiervan is.

Op basis van de bovenstaande resultaten zou geconcludeerd kunnen worden dat er geen leeftijds-geassocieerde afname is van de hoeveelheid DNA-herstel. Men moet zich echter realiseren dat hier maar twee verschillende typen DNA-schades onderzocht zijn, die beide volgens hetzelfde "long-patch" DNA-herstel mechanisme verwijderd worden. Uit de literatuur blijkt echter dat ook de verwijdering van DNA-breuken geïnduceerd door gammastraling, die via een ander (het zgn "short-patch") herstel mechanisme verwijderd worden, niet afneemt met de leeftijd.

Ondanks het feit dat de hoeveelheid DNA-herstel niet lijkt af te nemen met de leeftijd, bleek uit onze resultaten (hoofdstuk 2 en 3) dat zeker niet alle schade verwijderd wordt. In rattehuidcellen in vivo bijvoorbeeld was ongeveer 40 % van de pyrimidine dimeren nog aanwezig 24 uur na bestraling met ultraviolet licht, terwijl in levercellen 21 dagen na behandeling van de rat met AAF nog steeds 30 % van de schades aanwezig was.

De beste manier echter om aan te tonen dat DNA-schades zich ophopen tijdens het ouder worden, is het meten van de hoeveelheid "spontaan" aanwezige DNA-schade in verschillende organen van jonge en oude dieren. Een groot probleem hierbij is het gebrek aan kennis omtrent de meest relevante schade in dit opzicht. Uit de literatuur kan worden afgeleid dat enkelstrengs DNA-breuken en "AP-sites" (plaatsen in het DNA waar een suikergroep van diens base ontdaan is) met een hoge frequentie "spontaan", door b.v. lichaamswarmte of zuurstofradicalen, worden geïnduceerd (hoofdstuk 1). Verder is door Ames (1983) gesuggereerd dat carcinogene stoffen in het voedsel een belangrijke bron van DNA-schades zijn. Uit resultaten beschreven in hoofdstuk 5 bleek dat benzo(a)pyreen (BaP), een carcinogeen dat voorkomt in sigarettenrook maar soms ook in het voedsel, DNA-schades induceert in rattelever en darmcellen. Deze schade was alleen te detecteren met behulp van de

gevoelige alkalische elutie methode, waarmee zowel DNA-breuken als DNA-schades (bv AP-sites) die gedurende de behandeling met alkalische oplossingen omgezet worden in een DNA-breuk, gedetecteerd kunnen worden. Opvallend was dat deze BaP geinduceerde DNA-schades alleen gevonden werden in cellen die het BaP kunnen metaboliseren, zoals parenchymale levercellen en darmcellen en niet in de niet-parenchymale cellen, waarin geen metabolisatie plaats vindt. Bepalingsmethoden die alleen de inductie of verwijdering van grote, zgn "bulky" DNA-adducten detecteren leverden allen negatieve resultaten op. Nu is uit de literatuur bekend dat tijdens de metabolisatie van BaP radicalen kunnen vrijkomen die DNA-schades veroorzaken. Daarom is door ons gesuggereerd dat deze radicalen verantwoordelijk zijn voor de inductie van de gevonden DNA-breuken en alkalisch labiele schades, i.p.v. het carcinogeen zelf.

Uit het bovenstaande kan geconcludeerd worden dat zowel DNA-breuken als alkali-labiele plaatsen, DNA-schades zijn die met veroudering zouden kunnen accumuleren. Daarom werd onderzocht of er inderdaad een leeftijds geassocieerde ophoping van dit type schades optreedt in lever en hersencellen van de rat. Uit deze studies bleek dat in levercellen geïsoleerd uit oude ratten ongeveer twee-maal zoveel DNA-schades (700 per cel) aanwezig waren dan in levercellen uit jonge ratten (hoofdstuk 6). Opvallend hierbij was het feit dat deze verouderingsgerelateerde toename van het aantal DNA-schades alleen gevonden werd in de niet-delende (postmitotische) parenchymcellen. In de niet-parenchymale levercellen, die nog kunnen delen, bleef het aantal DNA-schades constant gedurende het ouder worden. Dit suggereert dat een verouderingsgeassocieerde toename in DNA-schades alleen optreedt in postmitotische cellen. Echter in rattehersenen, waarin zich ook een groot aantal postmitotische cellen bevinden, werd geen veroudering-geassocieerde toename in de hoeveelheid DNA-schades gevonden (Hoofdstuk 7).

Het feit dat de toename in hoeveelheid DNA-schade met de leeftijd alleen gevonden werd in postmitotische levercellen en niet in hersen cellen

suggereert dat deze schade geïnduceerd wordt door een lever-specifiek proces. De lever speelt een belangrijke rol bij de metabolisatie en detoxificatie van in het voedsel aanwezige carcinogene stoffen. Uit eerdere resultaten (hoofdstuk 5) is inderdaad al gebleken dat de carcinogene stof BaP DNA schades induceert, waarschijnlijk via radicalen die ontstaan tijdens de metabolisatie.

Het versneld afsterven en degenereren van hersencellen bij Alzheimer patienten zou volgens Robbins (1983) veroorzaakt kunnen worden door een verhoogd niveau van DNA-schades als gevolg van een defect in een of meer DNA-herstel systemen. Om deze hypothese te testen is de hoeveelheid DNA-schade gemeten, m.b.v. de alkalische elutie methode, in de hersenen (cortex) van Alzheimer patienten en van controles van dezelfde leeftijd. Uit de resultaten bleek dat het niveau van DNA-schades snel toenam naarmate de postmortem tijd (tijd tussen het overlijden en het beschikbaar komen van het hersen-materiaal) langer werd. Een soortgelijk fenomeen werd ook waargenomen in rattehersenen. Echter, na correctie voor het postmortem effect bleek dat in de hersenen van Alzheimer patienten ongeveer tweemaal zoveel DNA-beschadigingen aanwezig waren dan in de hersenen van controle-individuen. Een vergelijkbaar verschil in hoeveelheid DNA-schades tussen Alzheimer patienten en controles werd waargenomen wanneer alleen hersenmonsters met ongeveer dezelfde postmortem tijd vergeleken werden. Alhoewel dit in overeenstemming is met de hierboven genoemde hypothese van Robbins (1983) kan dit verhoogd schadeniveau ook het gevolg zijn van de ziekte. Op grond van onze resultaten lijkt het echter niet waarschijnlijk dat het grote aantal dode en gedegenereerde cellen, karakteristiek voor de ziekte van Alzheimer, de oorzaak is voor het hoge aantal DNA-breuken. Echter, een toename van de hoeveelheid geïnduceerde schade veroorzaakt door bijvoorbeeld een storing in een cellulair metabolisme is zeer wel mogelijk. Zo zijn er aanwijzingen dat er een versnelde oxidatie van vetten plaats vindt in de hersenen van Alzheimer patienten hetgeen zou kunnen leiden tot een toename in het niveau van radicalen.

Samengevat kunnen uit de in dit proefschrift beschreven resultaten de volgende conclusies getrokken worden:

1) Er is een duidelijk verschil in het percentage herstel van pyrimidine dimeren tussen rattehuidcellen in vitro en in vivo.
2) Er is geen algemene afname in de hoeveelheid DNA-herstel in rattecellen, noch in vitro noch in vivo, in relatie tot veroudering.
3) Een aanzienlijk deel van de schade geïnduceerd in vivo is gedurende lange tijd aanwezig in het DNA
4) Er is een leeftijdsgeassocieerde toename in de hoeveelheid DNA-schades in de postmitotische parenchymale levercellen maar niet in de nog delende niet-parenchymale levercellen en in hersencellen van de rat.
5) Het DNA uit de hersenen (cortex) van Alzheimer patienten bevat ongeveer twee maal zoveel DNA-schades als dat van controle individuen.

ABBREVIATIONS:

AAF	2-acetylaminofluorene
Aba	3-aminobenzamide
AD	Alzheimer's disease
ADPRT	ADP-ribosyl transferase
AE	alkaline elution
AF	N-deoxyguanosin(8-yl)aminofluorene
AP-site	apurinic site
AS	alkaline sucrose gradient sedimentation
AZC	alkaline zonal centrifugation
BaP	benzo(a)pyrene
BPDE	Benzo(a)pyrene diol epoxide
CG	cesium chloride gradient sedimentation;
DMEM	Dulbecco's Modification of Eagle's Medium
dpc	days post-coital
DPTA	DNA polymerase template activity
DSB	double-strand breaks
EB	ethidium bromide
ELISA	Enzyme-linked immonosorbent assay
ESS	UV-endonuclease sensitive sites
FCS	fetal calf serum
HM	hydrodynamic measurement
hprt	hypoxanthine phosphoribosyl transferase
IF	immunofluorescence with anti-cytidine antibody
mw	molecular weight
NS	nucleoid sedimentation
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
Pyr	pyrimidine dimer
S_1N	S_1 nuclease sensitivity
SOD	superoxide dismutase
SSB	single-strand breaks
TBA	tetrabutyl-ammonium chloride
UDS	unscheduled DNA synthesis
UV-C	ultraviolet light of the wavelength 254 nm.
UV-B	ultraviolet light of the wavelength range 290-320 nm.
UV	ultraviolet light
XP	xeroderma pigmentosum

REFERENCES:

Acharya, P.V.N. (1972) The isolation and partial characterization of age correlated oligodeoxyribo-ribonucleotide with covalently linked aspartylglutamyl-polypeptides. John Hopkins Med. J. Suppl., 1, 254-260.

Adelman, R., Saul, R.L. and Ames, B.N. (1988) Oxidative damage to DNA: Relation to species metabolic rate and life span. Proc. Natl. Acad. Sci. USA., 85, 2706-2708.

Ahnström, G. (1988) Techniques to measure DNA single-strand breaks in cells: A review. Int. J. Radiat. Biol., 54, 695-707.

Albertini, R.J., Castle, K.S. and Borcherding, W.R. (1982) T cell cloning to detect the mutant 6-thioguanine resistant lymphocytes present in human peripheral blood. Proc. Natl. Acad. Sci. USA., 79, 6617-6621.

Albertini, R.J., Sullivan, L.M., Berman, J.K., Greene, C.J., Stewart, J.A., Silveira, J.M. and O'Neill, J.P. (1988) Mutagenicity monitoring in humans by autoradiographic assay for mutant T lymphocytes. Mutation Res., 204, 481-492.

Ames, B.N. (1983) Dietary carcinogens and anticarcinogens. Science, 221, 1256-1264.

Aune, T., Vanderslice, R.R., Croft, J.E., Dybing, E., Bend, J.R. and Philpot, R.M. (1985) Deacetylation to 2-aminofluorene as a major initial reaction in the microsomal metabolism of 2-acetylaminofluorene to mutagenic products in preparations from rabbit lung and liver. Cancer Res., 45, 5859-5866.

Ashburner, M. and Bonner, J.J. (1979) The induction of gene activity in Drosophila by heat shock. Cell, 17, 241-254.

Baan, R.A., Van der Berg, P.T.M., Steenwinkel, M-J.S.T. and Van der Wulp, C.J.M. (1988) Detection of benzo(a)pyrene-DNA adducts in cultured cells treated with benzo(a)pyrene diol-epoxide by quantitative immunofluorescence microscopy and ^{32}P-postlabelling: Immunofluorescence analysis of benzo(a)pyrene-DNA adducts in bronchial cells from smoking individuals. in: Bartsch, H., Hemminki, K. and O'Neill, I.K. (Eds.), Methods for Detecting DNA Damaging Agents in Humans: Application in Cancer Epidemiology and Prevention, IARC Scientific publications, Lyon, pp. 146-151.

Bachelard, H.S., Hodder, V.E. and Walker, A.P. (1986) DNA damage and repair in Alzheimer's disease. in: Courtois, Y., Faucheux, B., Forette, B., Knook, D.L. and Tréton, J.A. (Eds.), Modern Trends in Aging Research, Eurage, Paris, pp. 451-458.

Bailly, V. and Verly, W.G. (1988) Possible roles of ß-elimination and δ-elimination reactions in the repair of DNA containing AP (apurinic/apyrimidinic) sites in mammalian cells. Biochem. J., 253, 553-559.

Balmain, A. (1981) Transforming ras oncogenes and multistage carcinogenesis. Br. J. Cancer, 51, 1-8.

Barrows, L.R. and Magee, P.N. (1982) Nonenzymatic methylation of DNA by S-adenosylmethionine in vitro. Carcinogenesis, 3, 349-351.

Beland, F.A., Dooley, K.L. and Jackson, C.D. (1982) Persistency of DNA adducts in rat liver and kidney after multiple doses of the carcinogen N-hydroxy-2-acetylaminofluorene. Cancer Res., 42, 1348-1354.

Benzi, G., Marzatico, F., Pastoris, O. and Villa, R.F. (1989) Relationship between aging, drug treatment and the cerebral enzymatic antioxidant system. Exp. Gerontol., 24, 137-148.

Boerrigter, M.E.T.I., Mullaart, E., Van der Schans, G.P. and Vijg, J. (1989a) Quiescent human peripheral blood lymphocytes do not contain a sizable amount of preexistent DNA single-strand breaks. Exp. Cell Res., 180, 569-573.

Boerrigter, M.E.T.I., Mullaart, E. and Vijg, J. (1989b) Induction and repair of DNA strand breaks in human lymphocytes exposed to N-ethyl-N-nitrosourea. Submitted for publication.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell, 40, 359-369.

Bojanovic, J.J., Jevtovic, A.D., Pantic, V.S., Dugandzic, S.M. and Jovanovic, D.S. (1970) Thymus nucleoproteins, thymus histones in young and adult rats. Gerontologia, 16, 304-312.

Boorstein, R.J., Levy, D.D. and Teebor, G.W. (1987) 5-Hydroxy-methyluracil-DNA glycosylase activity may be a differentiated function of mammalian cells. Mutation res., 183, 257-263.

Brash, D.E. and Hart, R.W. (1983) Fluorescent dye labeling to measure DNA damage in non-radiolabeled cells. in: Friedberg, E.C. and Hanawalt, P.C. (Eds.), DNA Repair: a Manual of Research Procedures, Vol 2, Dekker, New York, pp. 171-183.

Brooks, A., Mead, D. and Peters, R. (1973) Effect of aging on the frequency of metaphase chromosome aberrations in the liver of Chinese hamsters. J. Gerontol., 28, 452-454.

Brouns, R.E., Poot, M., Vrind, R., Hoek-Kon, T. and Henderson, P.T. (1979) Measurements of DNA-excision repair in suspensions of freshly isolated rat hepatocytes after exposure to some carcinogenic compounds. Mutation Res., 64, 425-432.

Brouwer, A., Barelds, R.J. and Knook, D.L. (1984) Centrifugal separation of mammalian cells. in: Rickwood, D. (Eds.), Centrifugation, A Practical Approach, IRL press, Oxford and Washington DC, pp. 183-218.

Brown, T.C. and Cerutti, P.A. (1987) Gene expression rather than the initiation of DNA replication is the principle target of lethal UV-induced damage in a regulatory region of SV40 DNA. Carcinogenesis, 8, 1133-1136.

Bucala, R., Model, P. and Cerami, A. (1984) Modification of DNA by reducing sugars: A possible mechanism for nucleic acid aging and age-related dysfunction in gene expression. Proc. Natl. Acad. Sci. USA., 81, 105-109.

Bucala, R., Model, P., Russel, M. and Cerami, A. (1985) Modification of DNA by glucose-6-phosphate induces DNA rearrangements in an E. coli plasmid. Proc. Natl. Acad. Sci. USA., 82, 8439-8442.

Burek, J.D. (1978) Pathology of aging rats. CRC press, West Palm Beach.

Cadet, J. and Berger, M. (1985) Radiation-induced decomposition of the purine bases within DNA and related model compounds. Int. J. Radiat. Biol., 47, 127-143.

Carle, G.F., Frank, M. and Olson, M.V. (1986) Electrophoretic separation of large DNA molecules by periodic inversion of the electric field. Science, 232, 65-68.

Carson, D.A., Seto, S., Wasson, D.B. and Carrera, C.J. (1986) DNA strand breaks, NAD metabolism, and programmed cell death. Exp. Cell Res., 164, 271-281.

Cathcart, R., Schwiers, E., Saul, R.L. and Ames, B.N. (1984) Thymine glycol and thymidine glycol in human and rat urine: A possible assay for oxidative DNA damage. Proc. Natl. Acad. Sci. USA., 81, 5633-5637.

Cedar, H. (1988) DNA methylation and gene activity. Cell, 53, 3-4.

Cerami, A. (1986) Aging of proteins and nucleic acids: what is the role of glucose? Trends in Biochemical Science, 11, 311-314.

Cerutti, P.A. (1985) Prooxydant states and tumor promotion. Science, 227, 375-381.

Cerutti, P.A., Emerit, I. and Amstad, P. (1983) Membrane-mediated chromosomal damage. in: Weinstein, J.B. and Vogel, H.J. (Eds.), Genes and Proteins in Oncogenesis, Academic Press, London, pp. 55-67.

Chaturvedi, M.M. and Kanungo, M.S. (1983) Analysis of chromatin of the brain of young and old rats by Micrococcal nuclease and DNase I. Biochemistry International, 6, 357-363.

Chetsanga, C.J., Boyd, V., Peterson, L. and Rushlow, K. (1975) Single-stranded regions in DNA of old mice. Nature, 253, 130-131.

Chetsanga, C.J., Tuttle, M. and Jacobini, A. (1976) Changes in structural integrity of heart DNA from aging mice. Life Sci., 18, 1405-1412.

Chetsanga, C.J., Tuttle, M., Jacobini, A. and Johnson, C. (1977) Age-associated structural alteration in senescent mouse brain DNA. Biochim. Biophys. Acta, 474, 180-187.

Cleaver, J.E. and Bootsma, D. (1975) Xeroderma pigmentosum: biochemical and genetic characteristics. Ann. Rev. Genet., 9, 19-38.

Cleaver, J.E. (1980) DNA damage, repair systems and human hypersensitive diseases. J. Environmental Pathology and Toxicology, 3, 53-68.

Coleman, P.D. and Flood, D.G. (1987) Neuron numbers and dendritic extent in normal and Alzheimer's disease. Neurobiol. Aging, 8, 521-545.

Crapper McLachlan, D.R., Lukiw, W.L., Wong, L., Bergeron, C. and Bech-Hansen, N.T. (1988) Selective messenger RNA reduction in Alzheimer's disease. Molecular Brain Res., 3, 255-262.

Crapper McLachlan, D.R., Lewis, P.N., Lukiw, W.J., Sima, A., Bergeron, C. and De Boni, U. (1984) Chromatin structure in dementia. Ann. Neurology, 15, 329-334.

Crine, P. and Verly, W.G. (1976) A study of DNA spontaneous degradation. Biochim. Biophys. Acta, 442, 50-57.

Crowley, C. and Curtis, H.J. (1963) The development of somatic mutations in mice with age. Proc. Natl. Acad. Sci. USA., 49, 626-628.

Curtis, H.J. and Miller, K. (1971) Chromosomal aberrations in liver cells of guinea pigs. J. Gerontol., 26, 292-293.

Cutler, R.G. (1984) Antioxidants, aging and longevity. in: Pryor, W. (Eds.), Free Radicals in Biology, Vol VI, Academic press, pp. 371-427

Cutler, R.G. (1985) Disdifferentiation hypothesis of aging: a review. in: Sohal, R.S., Birnbaum, L.S. and Cutler, R.G. (Eds.), Molecular Biology of Aging: Gene Stability and Expression, Raven Press, New York, pp. 307-340.

Cutler, R.G. (1986) Aging and oxygen radicals. in: Taylor, A.E., Matelon, S and Ward, P. (Eds.), Physiology of Oxygen Radicals, Am. Physiol. Soc., Bethesda, pp. 251-285.

D'Ambrosio, S.M., Slazinski, L., Whetstone, J.W. and Lowney, E. (1981) Excision repair of UV-induced pyrimidine dimers in human skin in vivo. J. Invest. Dermatol., 77, 311-313.

De Arce, M.A. (1981) The effect of donor sex and age on the number of sister chromatid exchanges in human lymphocytes growing in vitro. Human Genetics, 57, 83-85.

De Leeuw, A.M., Van Bezooijen, C.F.A. and Knook, D.L. (1986) Changes in the ratio between sinusoidal and parenchymal cell numbers of rat liver during development and aging. in: Kirn, A., Knook, D.L. and Wisse, E. (Eds.), Cells of the Hepatic Sinusoid, Vol. 1, Kupffer Cell Foundation, pp. 463-469.

Dean, R.G. and Cutler, R.G. (1978) Absence of significant age-dependent increase of single-stranded DNA extracted from mouse liver nuclei. Exp. Gerontol., 13, 287-292.

Drinkwater, R.D., Blake, T.J., Morley, A.A. and Turner, D.R. (1989) Human lymphocytes aged in vivo have reduced levels of methylation in transcriptionally and inactive DNA. Mutation Res., 219, 29-38.

Dutkowski, R.T., Lesh, R., Staiano-Coico, L., Thaler, H., Darlington, G.J. and Weksler, M.E. (1985) Increased chromosomal instability in lymphocytes from elderly humans. Mutation Res., 149, 505-512.

Eggset, G., Volden, G. and Krokan, H. (1983) UV-induced DNA damage and its repair in human skin in vivo studied by sensitive immunohistochemical methods. Carcinogenesis, 4, 745-750.

Eggset, G., Kavli, G., Krokan, H. and Volden, G. (1984) Ultraviolet and visible light penetration of epidermis. Photobiochem. Photobiophys., 8, 163-167.

Eker, A.R.M. (1983) Photorepair processes. in: Montagnoli, G. and Erlanger, B.F. (Eds.), Molecular Models of Photoresponsiveness, Plenum Press, New York, pp. 109-132.

Engvall, E. (1980) Enzyme immunoassay ELISA and EMIT. in: Van Vunakis, H. and Langone, J.J. (Eds.), Methods in Enzymology, Vol. 70, part A, Immunochemical Techniques, Academic press, New York, pp. 419-439.

Everson, R.B., Randerath, E., Santella, R.M., Cefalo, R.C., Avitts, T.A. and Randerath, K. (1986) Detection of smoking-related covalent DNA adducts in human placenta. Science, 231, 54-57.

Evans, H.J. and Vijayalaxmi (1981) Induction of 8-azaguanine resistance and sister chromatid exchanges in human lymphocytes exposed to mitomycin C and X-rays in vitro. Nature, 292, 601-605.

Farooq, M. and Norton, W.T. (1978) A modified procedure for isolation of astrocyte- and neuron-enriched fraction from rat brain. J. Neurochem., 31, 887-894.

Farzaneh, F., Zalin, R., Brill, D. and Shall, S. (1982) DNA strand breaks and ADP-ribosyl transferase activation during cell differentiation. Nature, 300, 362-366.

Farzaneh, F., Meldrum, R. and Shall, S. (1987) Transient formation of DNA strand breaks during induced differentiation of a human promyelocytic leukaemic cell line, HL-60. Nucleic Acids Research, 15, 3493-3502.

Featherstone, T., Marshall, P.D. and Evans, H.J. (1987) Problems and pitfalls in assessing human T-lymphocyte mutant frequencies. Mutation Res., 179, 215-230.

Finch, C.E. (1979) Susceptibility of mouse liver DNA to digestion by S1 nuclease: absence of age-related change. Age, 2, 45-47.

Fornace, A.J.Jr., Alama, I. and Hollander, M.C. (1988) DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. USA., 85, 8800-8804.

Freeman, B.A. and Crapo, J.D. (1982) Biology of disease. Free radicals and tissue injury. Lab. Invest., 47, 412-426

Fridovich, I. (1978) The biology of oxygen radicals: the superoxide radical is an agent of oxygen toxicity; superoxide dismutase provides an important defense. Science, 201, 875-880.

Friedberg, E.C. (1985) DNA repair, Freeman and Company, New York.

Ganesan, A.K., Spivak, G. and Hanawalt, P.C. (1983) Expression of DNA repair genes in mammalian cells. in: Nagley, P., Linnane, A.W., Peacock, W.J. and Pateman, J.A. (Eds.), Manipulation and Expression of Genes in Eukaryotes, Academic press, Sydney, pp. 45-54.

Gaubatz, J.W. (1989) Postlabeling analysis of indigenous aromatic DNA adducts in mouse myocardium during aging. Arch. Gerontol. Geriatr., 8, 47-54.

Gelboin, H.V. (1980) Benzo(a)pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxydases and related enzymes. Physiol. Rev., 60, 1107-1166.

Gensler, H.L. and Bernstein, H. (1981) DNA damage as the primary cause of aging. Quart. Rev. Biol., 56, 279-303.

Gensler, H.L., Hall, J.D. and Bernstein, H. (1987) The DNA damage hypothesis of aging: Importance of oxidative damage. in: Rothstein, M. (Eds.), Review of Biological Research in Aging, Vol. 3, Liss, New York, pp. 451-465.

Gilchrest, B.A. (1979) Relationship between actinic damage and chronological aging in keratinocyte cultures of human skin. J. Invest. Dermatol., 72, 219-223.

Gossen, J.A., De Leeuw, W.J.F., Tan, C.H.T., Zwarthoff, E.C., Berends, F., Lohman, P.H.M., Knook, D.L. and Vijg, J. (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a new model for studying mutations in vivo. Proc. Natl. Acad. Sci. USA., in press.

Greer, W.L. and Kaplan, J.G. (1983) DNA strand breaks in murine lymphocytes: Induction by purine and pyrimidine analogues. Biochem. Biophys. Res. Commun., 115, 834-840.

Gupta, R.C. (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen-DNA-adducts. Cancer Res., 45, 5656-5662.

Gupta, P.K. and Sirover, M.A. (1980) Sequential stimulation of DNA repair and DNA replication in normal human cells. Mutation Res., 72, 273-284.

Gupta, R.C., Reddy, M.V. and Randerath, K. (1982) ^{32}P-postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. Carcinogenesis, 3, 1081-1092.

Gupta, R.C., Dighe, N.R., Randerath, K. and Smith, H.C. (1985) Distribution of initial and persistent 2-acetylaminofluorene-induced DNA-adducts within loops. Proc. Natl. Acad. Sci. USA., 82, 6605-6608.

Hahn, G.M., King, D. and Yang, S.J. (1971) Quantitative changes in unscheduled DNA synthesis in rat muscle cells after differentiation. Nature New Biology, 230, 242-244.

Hall, J.D., Almy, R.E. and Scherer, K.L. (1982) DNA repair in cultured human fibroblasts does not decline with donor age. Exp. Cell Res., 139, 351-359.

Hanawalt, P.C. (1987) On the role of DNA damage and repair processes in aging: Evidence for and against. in: Warner, H.R., Butler, R.N., Sprott, R.L. and Schneider, E.L. (Eds.), Modern Biological Theories of Aging, Raven Press, New York, pp. 183-198.

Hardy, J.A., Wester, P., Winblad, B., Gezelius, C., Bring, G. and Eriksson, A. (1985) The patients dying after long terminal phase have acidotic brains; Implications for biochemical measurements on autopsy material. Neural Transmission, 61, 253-264.

Hariharan, P.V. and Cerutti, P.A. (1972) Formation and repair of gamma-ray-induced thymine damage in Micrococcus radiodurans. J. Mol. Biol., 66, 65-81.

Harman, D. (1981) The aging process. Proc. Natl. Acad. Sci. USA., 78, 7124-7181.

Harris, C.C., Autrup, H., Vahakangas, K. and Trump, B.F. (1984) Interindividual variation in carcinogen activation and DNA repair. in: Ommen, G.S. and Gelboin, H.V. (Eds.), Banbury Report 16, Genetic Variability in Response to Chemical Exposure, pp 145-153.

Harris, C.C., Vahakangas, K., Newman, M.J., Trives, G.E., Shamsuddin, A., Sinopoli, N., Mann, D.L. and Wright, W.E. (1985) Detection of benzo(a)pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. Proc. Natl. Acad. Sci. USA., 82, 6672-6676.

Harrison, D.D. and Webster, H.L. (1969) Isolation of crypt cells from the rat intestine. Exp. Cell Res., 55, 257-260.

Hart, R.W. and Setlow, R.B. (1974) Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species. Proc. Natl. Acad. Sci. USA., 71, 2169-2173.

Hart, R.W., D'Ambrossio, S.M., Ng, K.J. and Modak, S.P. (1979) Longevity, stability and DNA repair. Mech. Ageing Dev., 9, 203-223.

Hartwig, M. and Körner, I.J. (1987) Age-related changes of DNA winding and repair in human peripheral lymphocytes. Mech. Ageing Dev., 38, 73-78.

Hartnell, J.M., Storrie, M.C. and Mooradian, A.D. (1989) The tissue specificity of the age-related changes in alkali-induced DNA unwinding. Mutation Res., 219, 187-192.

Haseltine, W.A. (1983) Ultraviolet light repair and mutagenesis revisited. Cell, 33, 13-17.

Haugan, A., Becher, G., Benestad, C., Vahakangas, K., Trivers, G.E., Newman, M.J. and Harris, C.C. (1986) Determination of polycyclic aromatic hydrocarbons in the urine, benzo(a)pyrene diol epoxide-DNA adducts in lymphocyte DNA, and antibodies to the adducts in sera from coke oven workers exposed to measured amounts of polycyclic aromatic hydrocarbons in the work atmosphere. Cancer Res., 46, 4178-4183.

Hayflick, L. (1980) Recent advances in the cell biology of aging. Mech. Ageing Dev., 14, 59-79.

Hendriks, H.F.J., Verhoofstad, W.A.M.M., Brouwer, A., De Leeuw, A.M. and Knook, D.L. (1985) Perisinusoidal fat-storing cells are the main vitamin A storage sites in rat liver. Exp. Cell Res., 160, 138-149.

Hennen, L.A. (1987) Dosis van natuurlijke straling. in: Broerse, J.J., Hennen, L.A., Hermens, A.F. and Zoetelief, J. (Eds.), Grondbeginselen Stralingsfysica en Radiobiology voor Medische Toepasingen, Leiden, Rijswijk, pp. 187-200.

Hennis III, H.L., Braid, H.L. and Vincent, R.A. (1981) Unscheduled DNA synthesis in cells of different shape in fibroblast cultures from donors of various ages. Mech. Ageing Dev., 16, 355-361.

Higgins, S.A., Frenkel, K., Cummings, A. and Teebor, G.W. (1987) Definitive characterization of human thymine glycol N-glycosylase activity. Biochemistry, 26, 1683-1688.

Hollstein, M.C., Brooks, P., Linn, S. and Ames, B.N. (1984) Hydroxy-methyluracil DNA glycosylase in mammalian cells. Proc. Natl. Acad. Sci. USA., 81, 4003-4007.

Horn, P.L., Turker, M.S., Ogburn, G.E., Disteche, C.M. and Martin, G.M. (1984) A cloning assay for 6-thioguanine resistance provides evidence against certain somatic mutational theories of aging. J. Cell Physiol., 121, 309-315.

Hothersall, J.J., Greenbaum, A.L. and McLean, P. (1982) The functional significance of the pentose phosphate pathway in synaptosomes: Protection against peroxidative damage by catecholamines and oxidants. J. Neurochem., 39, 1325-1332.

Howard, J.W. and Fazio, T. (1980) Review of polycyclic aromatic hydrocarbons in foods. J. Assoc. Off. Anal. Chem., 63, 1077-1104.

Hsu, I.S., Harris, C.C., Lipsky, M.M., Snyder, S. and Trump, B.F. (1987) Cell and species difference in metabolic activation of chemical carcinogens. Mutation Res., 177, 1-7.

Huitfeldt, H.S., Spangler, E.F., Baron, J. and Poirier, M. (1987) Microfluorometric determination of DNA adducts in immunofluorescent-stained liver tissue from rats fed 2-acetylaminofluorene. Cancer Res., 47, 2098-2102.

IARC report, (1988) IARC Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Humans, Man-made Mineral Fibers and Radon, IARC, Lyon, pp. 173-241.

Ide, M.L., Kaneko, M. and Cerutti, P.A. (1983) Benzo(a)pyrene and ascorbate-$CuSO_4$ induced DNA damage in human cells by indirect action. in: McBrien, D.C.H. and Slater, T.F. (Eds.), Protective Agents in Cancer. Academic Press, London, pp. 125-140.

Ishikawa, T. and Sakurai, J. (1986) In vivo studies on age dependency of DNA repair with age in mouse skin. Cancer Res., 46, 1344-1348.

Iverson, L.L. (1979) The chemistry of the brain. Sci. Amer., 241, 118-129.

Joenje, H. (1989) Genetic Toxicology of Oxygen. Mutaion Res., in press.

Jostes, R., Reese, J.A., Cleaver, J.E., Molero, M. and Morgan, W.F. (1989) Quiescent human lymphocytes do not contain DNA strand breaks detectable by alkaline elution. Exp. Cell Res., 182, 513-520.

Johnstone, A.P. and Williams, G.T. (1982) Role of DNA breaks and ADP-ribosyl transferase activity in eukaryotic differentiation demonstrated in human lymphocytes. Nature, 300, 368-370.

Kantor, G.J. and Setlow, R.B. (1981) Rate and extent of DNA repair in nondividing human diploid fibroblasts. Cancer Res., 41, 819-825.

Karran, P. and Ormerod, M.G. (1973) Is the ability to repair damage to DNA related to the proliferative capacity of the cell ? The rejoining of X-ray-produced strand breaks. Biochim. Biophys. Acta, 299, 54-64.

Karran, P. and Lindahl, T. (1980) Hypoxanthine in deoxyribonucleic acid: Generation by heat-induced hydrolysis of adenine residues in free form by a deoxyribonucleic acid glycosylase from calf thymus. Biochemistry, 19, 6005-6011.

Kastan, M.B., Gowans, B.J. and Lieberman, M.W. (1982). Methylation of deoxycytidine incorporated by excision-repair synthesis of DNA. Cell, 30, 509-516.

Kato, H., Harada, M., Tsuchiya, K. and Moriwaki, K. (1980) Absence of correlation between DNA repair in ultraviolet irradiated mammalian cells and life-span of the donor species. Japan J. Genetics, 55, 99-108.

Kato, T. and Kurokawa, M. (1967) Isolation of cell nuclei from the mammalian cerebral cortex and their assortment on a morphological basis. J. Cell Biol., 32, 649-662.

Kempf, C., Schmitt, M., Danse, J. and Kempf, J. (1984) Correlation of DNA repair synthesis with ageing in mice, evidenced by quantitative autoradiography. Mech. Ageing Dev., 26, 183-194.

Keyse, S.M. and Tyrell, R.M. (1987) Both near ultraviolet radiation and the oxidizing agent hydrogen peroxide induce a 32-kDa stress protein in normal human skin fibroblasts. J. Biol. Chem., 262, 14821-14825.

Kidson, C. and Dambergs, R. (1982) Nervous system development and ataxia-telangiectasia. in: Bridges, B.A. and Harnden, D.G. (Eds.), A Cellular and Molecular Link between Cancer, Neuropathology and Immune Deficiency, John Wiley and Sons, Chichester, pp. 373-377.

Kinsella, T.J., Dobson, P.P., Fornace, A.J., Barret, S.F., Ganges, M.B. and Robbins, J.H. (1987) Alzheimer's disease fibroblasts have normal repair of N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA damage determined by the alkaline elution technique. Biochem. Biophys. Res. Commun., 149, 355-361.

Kinsella, T.J., Dobson, P.P., Fornace, A.J., Ganges, M.B., Barret, S.F. and Robbins, J.H. (1987) Alzheimer's disease fibroblasts have normal repair of methylmethane sulfonate-induced DNA damage determined by the alkaline elution technique. Neurology, 37, (supl. 1), 166.

Kirkwood, T.B.L. (1989) DNA, mutations and aging. Mutation Res., 219, 1-7.

Kirkwood, T.B.L. and Cremer, T. (1982) Cytogerontology since 1881: a reappraisal of August Weissman and a review of modern progress. Hum. Genet., 60, 101-121.

Kirkwood, T.B.L. and Holliday, R. (1986) Selection for optimal accuracy and the evolution of ageing. in: Kirkwood, T.B.L., Rosenberger, R.F. and Galas, D.J. (Eds.), Accuracy in Molecular Processes, Chapman and Hall, London, New York, pp. 363-380.

Knook, D.L. (1980) The isolated hepatocyte: a cellular model for aging studies, Proc. Soc. Exp. Biol. Med., 165, 170-177.

Knook, D.L. and Hollander, C.F. (1978) Embryology and aging of the rat liver. in: Newberne, P.M. and Butler, W.H. (Eds.), Rat Hepatic Neoplasia, MIT Press, pp. 8-40.

Kodamo, K., Ishikawa, T. and Takayama, S. (1984) Dose response, wavelength dependency, and time course of ultraviolet radiation-induced unscheduled DNA synthesis in mouse skin in vivo. Cancer Res., 44, 2150-2154.

Kohn, K.W., Erickson, L.C., Ewig, R.A.G. and Friedman, C.A. (1976) Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry, 15, 4629-4637.

Konze-Thomas, B., Hazard, R.M., Maher, V.M. and McCormick, J.J. (1982) Extent of excision repair before DNA synthesis determines the mutagenic but not the lethal effect of UV radiation. Mutation Res., 94, 421-434.

Kriek, E. and Westra, J.G. (1980) Structural identification of the pyrimidine derivatives formed from N-(deoxyguanosin-8-yl)-2-aminofluorene in aqueous solution at alkaline pH+. Carcinogenesis, 1, 459-468.

Kurtz, D.I., Russell, A.P. and Sinex, F.M. (1974) Multiple peaks in the derivative melting curve of chromatin from animals of varying age. Mech. Ageing Dev., 3, 37-49.

La Belle, M. and Linn, S. (1984) DNA repair in cultured mouse cells of increasing population doubling level. Mutation Res., 132, 51-61.

Laher, J.M., Rigler, M.W., Vetter, R.D., Barrowman, J.A. and Patton, J.S. (1984) Similar bioavailability and lymphatic transport of benzo(a)pyrene when administered to rats in different amounts of dietary fat. J. Lip. Res., 25, 1337-1342.

Lawson, T. and Stohs, S. (1985) Changes in endogenous DNA damage in aging mice in response to butylated hydroxyanisole and oltripraz. Mech. Ageing Dev., 30, 179-185.

Leadon, S.A., Stampfer, M.R. and Bartley, J. (1988) Production of oxidative DNA damage during metabolic activation of benzo(a)pyrene in human mammary epithelial cells correlates with cell killing. Proc. Natl. Acad. Sci. USA., 85, 4365-4368.

Lee, A.T. and Cerami, A. (1987) The formation of reactive intermediate(s) of glucose 6-phosphate and lysine capable of rapidly reacting with DNA. Mutation Res., 179, 151-158.

Lesko, S.A. (1984) Chemical carcinogenesis: benzopyrene system. Methods in Enzymology, 105, 539-550.

Lewis, P.N., Lukiw, W.J., De Boni, U. and Crapper Mclachlan, D.R. (1981) Changes in chromatin structure associated with Alzheimer's disease. J. Neurochem., 37, 1193-1202.

Li, J.C. and Kaminskas, E. (1985) Deficient repair of DNA lesions in Alzheimer's disease. Biochem. Biophys. Res. Commun., 129, 733-738.

Lindahl, T. (1982) DNA repair enzymes. Ann. Rev. Biochem., 51, 61-87.

Lindahl, T. and Andersson, A. (1972) Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid. Biochemistry, 11, 3619-3622.

Lindahl, T. and Nyberg, B. (1972) Rate of depurination of native deoxyribonucleic acid. Biochemistry, 11, 3610-3618.

Lindahl, T. and Karlström, O. (1973) Heat-induced depyrimidination of deoxyribonucleic acid in neutral solution. Biochemistry, 12, 5151-5154.

Lindahl, T. and Nyberg, B. (1974) Heat-induced deamination of cytosine residues in deoxyribonucleic acid. Biochemistry, 13, 3405-3410.

Lindahl, T. (1977) DNA repair enzymes acting on spontaneous lesions in DNA. in: Nichols, W.W. and Murphy, D.G. (Eds.), DNA Repair Processes, Symposia Specialists Inc., Miami, pp. 225-240.

Liu, S.C., Meagher, K. and Hanawalt, P.C. (1985) Role of solar conditioning in DNA repair response and survival of human epidermal keratinocytes following UV irradiation. J. Invest. Dermatol., 85, 93-97.

Liu, S.C., Parsons, C.S. and Hanawalt, P.C. (1982) DNA repair response in human epidermal keratinocytes from donors of different age. J. Invest. Dermatol., 79, 330-335.

Loeb, L.A. and Preston, B.D. (1986) Mutagenesis by apurinic/apyrimidinic sites. Ann. Rev. Genet., 20, 201-230.

Loechler, E.D., Green, C.L. and Essigmann, J.M. (1984) In vivo mutagenesis by O^6-methylguanine built into a unique site in a viral genome. Proc. Acad. Natl. Sci. USA., 6271-6275.

Lohman, P.H.M., Paterson, M.C., Zelle, B. and Reynolds, R.J. (1976) DNA repair in Chinese hamster cells after irradiation with ultraviolet light. Mutation Res., 46, 138-139.

Lohman, P.H.M., Baan, R.A., Fichtinger-Schepman, A.M.J., Muysken-Schoen, M.A., Lansbergen, R.J. and Berends, F. (1985) Molecular dosimetry of genotoxic damage: biochemical and immunochemical methods to detect DNA damage. Trends in Pharmacological Science, 1-7.

Lohman, P.H.M., Vijg, J., Uitterlinden, A.G., Slagboom, P.S., Gossen, J.A. and Berends, F. (1987) DNA methods for detecting and analyzing mutations in vivo. Mutation Res., 181, 227-234.

Lonati-Galligani, M., Lohman, P.M.H. and Berends, F. (1983) The validity of the autoradiographic method for detecting DNA repair synthesis in rat hepatocytes in primary culture. Mutation Res., 113, 145-160.

Lorentzen, R.J. and Ts'o, P.O.P. (1977) Benzo(a)pyrenedione/ benzo(a)pyrenediol oxidation-reduction couples and the generation of reactive reduced molecular oxygen. Biochemistry, 16, 1467-1473.

Lutz, W.K. (1979) In vivo covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. Mutation. Res., 65, 289-356.

Marlhens, F., Achkar, W.Al., Aurias, A., Couturier, J., Dutrillaux, A.M., Gerbault-Sereau, M., Hoffschir, F., Lamoliatte, E., Lefrancois, D., Lombard, M., Muleris, M., Prieur, M., Prod'homme, M., Sabatier, L., Vieges-Péqiugnot, E., Volobouev, V. and Dutrillaux, B. (1986) The rate of chromosome breakage is age dependent in lymphocytes of adult controls. Hum. Genet., 73, 290-297.

Martin, G.M., Smith, A.C., Ketterer, D.J., Ogburn, C.E. and Disteche, C.M. (1985) Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. Isr. J. Med. Sci., 21, 296-301.

Martins, R.N., Harper, C.G., Stokes, G.B. and Masters, C.L. (1986) Increased cerebral glucose-6-phosphate dehydrogenase activity in Alzheimer's disease may reflect oxidative stress. J. Neurochem., 46, 1042-1045.

Massie, H.R., Baird, M.B., Nicholosi, R.J. and Sarris, H.B. (1972a) Changes in the structure of rat liver DNA in relation to age. Arch. Biochem. Biophys., 153, 736-741.

Massie, H.R., Samis, H.V. and Baird, M.B. (1972b) The kinetics of degradation of DNA and RNA by H_2O_2. Biochim. Biophys. Acta, 272, 539-548.

Mayne, L.V. and Lehmann, A.R. (1982) Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. Cancer Res., 42, 1473-1478.

McClanahan, T. and McEntee, K. (1986) DNA damage and heat shock dually regulate genes in Saccharomyces cerevisiae. Mol. Cell. Biol., 6, 90-96.

Meek, R.L., Rebeiro, T. and Daniel, C.W. (1980) Patterns of unscheduled DNA synthesis in mouse embryo cells associated with in vitro aging and with spontaneous transformation to a continuous cell line. Exp. cell Res., 129, 265-271.

Mellon, I., Spivak, G. and Hanawalt, P.C. (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell, 51, 241-249.

Meneghini, R., Menck, C.F.M. and Schumacher, R.I. (1981) Mechanisms of tolerance of DNA lesions in mammalian cells. Quart. Rev. Biophys., 14, 381-432.

Michalopoulos, G., Sattler, G.L., O'Connor, L. and Pilot, H.C. (1978) Unscheduled DNA synthesis induced by procarcinogens in suspensions and primary cultures of hepatocytes on collagen membranes. Cancer Res., 38, 1866-1871.

Miller, E.C. and Miller, J.A. (1974) Biochemical mechanisms of chemical carcinogenesis. in: Busch, H. (Eds.), The Molecular Biology of Cancer, Academic Press, New York, pp. 377-402.

Miller, E.C. (1978) Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. Cancer Res., 38, 1479-1496.

Moore, C.J. and Schwarz, A.G. (1978) Inverse correlation between species lifespan and the capacity of cultured fibroblasts to convert benzo(a)pyrene to water-soluble metabolites. Exp. Cell Res., 116, 359-364.

Morgan, W.F. and Crossen, P.E. (1977) The incidence of sister chromatid exchanges in cultured human lymphocytes. Mutation Res., 42, 305-312.

Mori, N. and Goto, S. (1982) Estimation of the single stranded region in the nuclear DNA of mouse tissues during aging with special reference to the brain. Arch. Gerontol. Geriatr., 1, 143-150.

Morley, A.A., Cox, S. and Holliday, R. (1982) Human lymphocytes resistant to 6-thioguanine increase with age. Mech. Ageing Dev., 19, 21-26.

Moustacchi, E., Ehmann, U.K. and Friedberg, E.C. (1979) Defective recovery of semi-conservative DNA synthesis in xeroderma pigmentosum cells following split-dose ultraviolet irradiation. Mutation Res., 62, 159-171.

Mullaart, E., Lohman, P.H.M. and Vijg, J. (1988a) Differences in pyrimidine dimer removal between rat skin cells in vitro and in vivo. J. Invest. Dermatol., 90, 346-349.

Mullaart, E., Boerrigter, M.E.T.I., Brouwer, A., Berends, F. and Vijg, J. (1988b) Age-dependent accumulation of DNA alkali-labile sites in DNA of post-mitotic but not in that of mitotic rat liver cells. Mech. Ageing Dev., 45, 41-49.

Mullaart, E., Roza, L., Lohman, P.H.M. and Vijg, J. (1989a) The removal of UV-induced pyrimidine dimers in rat skin cells in vitro and in vivo in relation to aging. Mech. Aging Dev., 47, 253-264.

Mullaart, E., Boerrigter, M.E.T.I., Lohman, P.H.M. and Vijg, J. (1989b) Age-related induction and disappearance of carcinogen-DNA-adducts in livers of rats exposed to low levels of 2-acetylaminofluorene. Chem. Biol. Interact., 69, 373-384.

Mullaart, E., Buytenhek, M., Brouwer, A., Lohman, P.H.M. and Vijg, J. (1989c) Genotoxic effects of intragastrically administered benzo(a)pyrene in rat liver and intestinal cells. Carcinogenesis, 10, 393-395.

Mullaart, E., Boerrigter, M.E.T.I., Boer, G.J. and Vijg, J. (1989d) The level of spontaneous DNA breaks in rat brain during development and aging. Submitted for publication.

Mullaart, E., Boerrigter, M.E.T.I., Ravid, R., Swaab, D.F. and Vijg, J. (1989e) Increased levels of DNA breaks in cerebral cortex of Alzheimer's disease patients. Submitted for publication

Mullenders, L.H.F., Van Kesteren van Leeuwen, A.C., Van Zeeland, A.A. and Natarajan, A.T. (1988) Nuclear matrix associated DNA is preferentially repaired in normal human fibroblasts, exposed to a low dose of ultraviolet light but not in Cockayne's syndrome fibroblasts. Nucleic Acid Research, 16, 10607-10622.

Müller, M.M., Gerster, T. and Schaffner, W. (1988) Enhancer sequences and the regulation of gene transcription. Eur. J. Biochem., 176, 485-495.

Nakanishi, K., Shima, A., Fukuda, M. and Fujita, S. (1979) Age associated increase of single-stranded regions in the DNA of mouse brain and liver. Mech. Ageing Dev., 10, 273-281.

Naqui, A., Chance, B. and Cadenas, E. (1986) Reactive oxygen intermediates in biochemistry. Ann. Rev. Biochem., 55, 137-166.

Natarajan, A.T. and Obe, G. (1978) Molecular mechanisms involved in the production of chromosomal aberrations. I. Utilization of Neurospora endonuclease for the study of aberration production in G2 stage of the cell cycle. Mutation Res., 52, 137-149.

Natarajan, A.T., Obe, G., Van Zeeland, A.A., Palitti, F., Meijers, M. and Verdegaal-Immerzeel, E.A.M. (1980) Molecular mechanisms involved in the production of chromosomal aberrations. II. Utilization of Neurospora endonuclease for the study of aberration production by X-rays in G1 and G2 stages of the cell cycle. Mutation Res., 69, 293-305.

Nero, A.V. (1988) Controlling indoor air pollution. Sci. Am., 258, 24-30.

Nette, E.G., Xi, Y., Sun, Y., Andrews, A.D. and King, D.W. (1984) A correlation between aging and DNA repair in human epidermal cells. Mech. Ageing Dev., 24, 283-292.

Niedermüller, H. (1982) Age dependency of DNA repair in rats after DNA damage by carcinogens. Mech. Ageing Dev., 19, 259-271.

Niedermüller, H., Hofecker, G. and Skalicky, M. (1985) Changes of DNA repair mechanisms during the aging of the rat. Mech. ageing Dev., 29, 221-238.

Niggli, H.J. and Röthlisberger, R. (1988) Sunlight-induced pyrimidine dimers in human skin fibroblasts in comparison with dimerization after artificial UV-irradiation. Photochem. Photobiol., 48, 353-356.

Ono, T. and Okada, S. (1973) An alkaline sucrose gradient centrifugation method applicable to non-dividing and slowly dividing cells. J. Radiat. Res., 14, 204-207.

Ono, T., Okada, S. and Sugahara, T. (1976) Comparative studies of DNA size in various tissues of mice during the aging process. Exp. Gerontol., 11, 127-132.

Ono, T. and Cutler, R.G. (1978) Age-dependent relaxation of gene expression: increase of endogenous murine leukemia virus-related and globin-related RNA in brain and liver of mice. Proc. Natl. Acad. Sci. USA., 75, 4431-4435.

Ono, T. and Okada, S. (1978) Does the capacity to rejoin radiation-induced DNA breaks decline in senescent mice? Int. J. Radiat. Biol., 33, 403-407.

Park, S.D. and Cleaver, J.E. (1979) Postreplication repair: questions of its definition and possible alteration in xeroderma pigmentosum cell strains. Proc. Natl. Acad. Sci. USA., 76, 3927-3931.

Paterson, M.C., Lohman, P.H.M. and Sluyter, M.L. (1973) Use of a UV-endonuclease from Micrococcus luteus to monitor the progress of DNA repair in UV-irradiated human cells. Mutation Res., 19, 245-256.

Peleg, L., Raz, E. and Ben-Ishai, R. (1977) Changing capacity for DNA excision repair in mouse embryonic cells in vitro. Exp. Cell Res., 104, 301-307.

Perera, F.P., Santella, R.M., Brenner, D., Poirier, M.C., Munshi, A.A., Fischman, H.K. and Van Ryzin, J. (1987) DNA adducts, protein adducts and sister chromatid exchange in cigarette smokers and nonsmokers. J. Natl. Cancer Inst., 79, 449-456.

Perera, F.P., Hemminski, K., Young, T.L., Brenner, D., Kelly, G. and Santella, R.M. (1988) Detection of polycyclic aromatic hydrocarbon-DNA adducts in white blood cells of foundry workers. Cancer Res., 48, 2288-2291.

Phillips, D.H., Hewer, A., Martin, C.N., Garner, R.C. and King, M.M. (1988a) Correlation of DNA adduct levels in human lung with cigarette smoking. Nature, 336, 790-792.

Phillips, D.H., Hemminki, K., Alhonen, A., Hewer, A. and Grover, P.L. (1988b) Monitoring occupational exposure to carcinogens: detection by ^{32}P-postlabelling of aromatic DNA adducts in white blood cells from iron foundry workers. Mutation Res., 204, 531-541.

Price, G.B., Modak, S.P. and Makinodan, T. (1971) Age-associated changes in the DNA of mouse tissue. Science, 171, 917-919.

Prieur, M., Achkar, W.Al., Auriar, A., Couturier, J., Dutrillaux, A.M., Dutrillaux, B., Flüry-Herard, A., Gerbault-Seureau, M., Hoffschir, F., Lamoliatte, E., Lefrancois, D., Lombard, M., Muleris, M., Ricoul, M., Sabatier, L. and Viegas-Péquignot, E. (1988) Acquired chromosome rearrangements in human lymphocytes: effect of aging. Hum. Genet., 79, 147-150.

Radman, M. (1975) SOS repair hypothesis. in: Hanawalt, P.C. and Setlow, R.B. (Eds.), Molecular Mechanisms for Repair of DNA, Plenum Press, New York, pp. 355-367.

Randerath, E., Avitts, T.A., Reddy, M.V., Miller, R.H., Everson, R.B. and Randerath, K. (1986a) Comparative ^{32}P-analysis of cigarette smoke-induced DNA damage in human tissues and mouse skin. Cancer Res., 46, 5869-5877.

Randerath, K., Reddy, M.V. and Disher, M. (1986b) Age-and tissue-related DNA modifications in untreated rats: detection by ^{32}P-postlabeling assay and possible significance for spontaneous tumor induction and aging. Carcinogenesis, 7, 1615-1617.

Randerath, K., Lu, L-J.W. and Donghui, L. (1988) A comparison between different types of covalent DNA modifications (I-compounds, persistent carcinogen adducts and 5-methyl-cytosine) in regenerating liver. Carcinogenesis, 9, 1843-1848.

Randerath, K., Liehr, J.G.., Gladek, A. and Randerath, E. (1989) Age-dependent covalent DNA alterations (I-compounds) in rodent tissues: species, tissue and sex specificities. Mutation Res., 219, 121-133.

Reddy, M.V. and Randerath, K. (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis, 7, 1543-1551.

Regan, J.D. and Setlow, R.B. (1974) Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. Cancer Res., 34, 3318-3325.

Repine, J.E., Pfenninger, O.W., Talmage, D.W., Berger, E.M. and Pettijohn, D.E. (1981) Dimethyl sulfoxide prevents DNA nicking mediated by ionizing radiation or iron/hydrogen peroxide-generated hydroxyl radicals. Proc. Natl. Acad. Sci. USA., 78, 1001-1003.

Rheinwald, J.C. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizating colonies from single cells. Cell, 6, 331-344.

Ribeiro, P.L., Martinez, M.C. and Mitra, R.S. (1985) A possible correlation between the growth rate and the extent of DNA damage induced by radiodecay in mouse lymphoma cells. Biochem. Biophys. Res. Commun., 128, 204-210.

Richardson, A., Birchenall-Sparks, M.C. and Staecker, J.L. (1983) Aging and transcription. in: Rothstein, M. (Eds.), Review of Biological Research in Aging, Vol. 1, Liss, New York, pp. 275-294.

Richter, C., Park, J-W. and Ames, B.N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc. Natl. Acad. Sci. USA., 85, 6465-6467.

Robbins, J.H. (1983) Hypersensitivity to DNA-damaging agents in primary degenerations of excitable tissue. in: Friedberg, E.C. and Bridges, B.A. (Eds.), Cellular Responses to DNA Damage, Liss, New York, pp. 671-700.

Robbins, J.H., Kraemer, K.H., Lutzner, M.A., Festoff, B.W. and Coon, H.G. (1974) Xeroderma pigmentosum: an inherited disease with sun sensitivity, multiple cutaneous neoplasms, and abnormal DNA repair. Ann. Intern. Med., 80, 221-248.

Robbins, J.H., Otsuka, F., Tarone, R.E., Polinsky, R.J., Brumback, R.A., Moshell, A.N., Nee, L.E., Ganges, M.B. and Cayeux, S.J. (1983) Radiosensitivity in Alzheimer's disease and Parkinson disease. Lancet, 1, 468-469

Robbins, J.H., Otsuka, F., Tarone, R.E., Polinsky, R.J., Brumback, R.A. and Nee, L.E. (1985) Parkinson's disease and Alzheimer's disease: hypersensitivity to X rays in cultured cell lines. J. Neurology, Neurosurgery and Psychiatry, 48, 916-923.

Robison, S.H., Munzer, J.S., Tandan, R. and Bradley, W.G. (1987) Alzheimer's disease cells exhibit defective repair of alkylating agents-induced DNA damage. Ann. Neurol., 21, 250-258.

Roots, R. and Okada, S. (1975) Estimation of life times and diffusion distances of radicals involved in X-ray-induced DNA strand breaks or killing of mammalian cells. Radiat. Res., 64, 306-320.

Roza, L., Van der Wulp, K.J.M., MacFarlane, S.J., Lohman, P.M.H. and Baan, R.A. (1988) Detection of cyclobutane thymine dimers in DNA of human cells with monoclonal antibodies raised against a thymine dimer-containing tetranucleotide. Photochem. Photobiol., 48, 627-633.

Rupp, W.D. and Howard-Flanders, P. (1968) Discontinuities in the DNA synthesized in an excision-deficient strain of Escherichia coli following ultraviolet irradiation. J. Mol. Biol., 31, 291-304

Rupp, W.D., Wilde, C.E., Reno, D.L. and Howard-Flanders, P. (1971) Exchanges between DNA strands in ultraviolet irradiated Escherichia coli. J. Mol. Biol., 61, 25-44.

Russell, A.P., Dowling, L.E. and Hermann, R.L. (1970) Age-related differences in mouse liver DNA melting and hydroxylapatite fractionation. Gerontologia, 16, 159-171.

Rydberg, B. and Lindahl, T. (1982) Nonenzymatic methylation of DNA by intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO J., 1, 211-216

Sajdel-Sulkowska, E.M. and Marotta, C.A. (1984) Alzheimer's disease brain: Alterations in RNA levels and in a ribonuclease-inhibitor complex. Science, 225, 947-948.

Saul, R.L. and B.N. Ames (1985) Background levels of DNA damage in the population. in: Simic, M., Grossman, L. and Upton, A. (Eds.), Mechanisms of DNA Damage and Repair, Plenum press, New York, pp. 529-536.

Saul, R.L., Gee, P. and Ames, B.N. (1987) Free radicals, DNA damage, and aging. in: Warner, H.R., Butler, R.N., Sprott, R.L. and Schneider, E.L. (Eds.), Modern Biological Theories of Aging, Raven press, New York, pp. 113-130.

Scarpa, M., Rigo, A., Viglino, P., Stevanato, R., Bracco, F. and Battistin, L. (1987) Age dependency of the level of the enzymes involved in the protection against active oxygen species in the rat brain. Proc. Soc. Exp. Biol. Med., 185, 129-133.

Schaaper, R.M. and Loeb, L.A. (1981) Depurination causes mutations in SOS-induced cells. Proc. Natl. Acad. Sci. USA., 78, 1773-1777.

Schmidt, M.A. and Sanger, W.G. (1981) Sister chromatid exchanges in aged human lymphocytes. A brief note. Mech. Ageing Dev., 16, 67-70.

Schneider, E.L. and Monticone, R.E. (1978) Aging and sister chromatid exchanges. II. The effect of the in vitro passage level of human fetal lung fibroblasts on baseline and mutagen-induced sister chromatid exchange frequencies. Exp. Cell Res., 115, 269-276.

Schneider, E.L., Bickings, C.K. and Sternberg, H. (1982) Aging and sister chromatid exchanges VII. Effect of aging on background SCE in vivo. Cytogenet. Cell Genet., 33, 249-253.

Schutte, H.H., Van der Schans, G.P. and Lohman, P.H.M. (1988) Comparison of induction and repair of adducts and of alkali-labile sites in human lymphocytes and granulocytes after exposure to ethylating agents. Mutation Res., 194, 23-37.

Schwarz, A.G. and Moore, C.J. (1977) Inverse correlation between species lifespan and capacity of cultured fibroblasts to bind 7,12-demethylbenz(a)anthracence to DNA. Exp. Cell Res., 109, 448-450.

Scudiero, D.A., Polinsky, R.J., Brumback, R.A., Tarone, R.E., Nee, L.E. and Robbins, J.H. (1986) Alzheimer disease fibroblasts are hypersensitive to lethal effects of DNA-damaging agents. Mutation Res., 159, 125-131.

Setlow, R.B. (1978) Repair deficient human disorders and cancer. Nature, 273, 713-718.

Setlow, R.B. (1982) DNA repair, aging and cancer. Natl. Cancer Inst. Monogr., 60, 249-255.

Setlow, R.B. (1983) Variations in DNA repair among humans. in: Harris, C.C. and Autrup, H.N. (Eds.), Human Carcinogenesis, Academic press, pp. 231-254.

Shamberger, R.J., Baughman, F.F., Kalchert, S.L., Willis, C.E. and Hoffman, G.C. (1973) Carcinogen-induced chromosomal breakage is decreased by antioxidants. Proc. Natl. Acad. Sci. USA., 70, 1461-1463.

Shamsuddin, A.K.M., Sinopoli, N.T., Hemminki, K., Boesch, R.R. and Harris, C.C. (1985) Detection of benzo(a)pyrene:DNA adducts in human white blood cells. Cancer Res., 45, 66-68.

Sharma, R.C. and Yamamoto, O. (1980) Base modification in adult animal liver DNA and similarity to radiation-induced base modification. Biochem. Biophys. Res. Commun., 96, 662-671.

Slagboom, P.E. and Vijg, J. (1989) Genetic instability and aging: theories, facts and future perspectives. Genome, in press

Slagboom, P.E., De Leeuw, W.J.F. and Vijg, J. (1989) Messenger RNA levels and methylation patterns of liver specific genes in aging inbred rats. Submitted for publication.

Sohal, R.S. and Allen, R.G. (1985) Relationship between metabolic rate, free radicals, differentiation and aging: A unified theory. in: Woodhead, A.D., Blackett, A.D. and Hollaender, A. (Eds.), The molecular Basis of Aging, Plenum Press, New York, pp. 75-104.

Spokes, E.G.S. and Koch, D.J. (1978) Post-mortem stability of dopamine, glutamate decarboxylase and choline acetyltransferase in the mouse brain under conditions simulating the handling of human autopsy material. J. Neurochem., 31, 381-383.

Stewart, S.S., McManaman, J.L., Smith, R.G., Tomozawa, Y., Bostwick, J.R. and Appel, S.H. (1988) The role of neurotrophic factors in the pathogenesis of neurologic disease. in: Strong, R. et al. (Eds.), Central Nervous System Disorders of Aging: Clinical Intervention and Research, Raven Press, New York, pp. 25-39.

Stout, D.L. (1980) Covalent binding of 2-acetylaminofluorene, 2-aminofluorene, and N-hydroxy-2-acetylaminofluorene to rat liver nuclear DNA and protein in vivo and in vitro. Cancer Res., 40, 3579-3584.

Stout, D.L. and Becker, F.F. (1982) Fluorometric quantitation of single-stranded DNA: A method applicable to the technique of alkaline elution. Anal. Biochem., 13, 302-307.

Strauss, G.H. and Albertini, R.J. (1979) Enumeration of 6-thioguanine-resistent peripheral blood lymphocytes in man as a potential test for somatic cell mutations arising in vivo. Mutation Res., 61, 353-379.

Strehler, B.L. (1986) Genetic instability as the primary cause of human aging. Exp. Gerontol., 21, 283-319.

Su, C.M., Brash, D.E., Turturro, A. and Hart, R.W. (1984) Longevity-dependent organ-specific accumulation of DNA damage in two closely related murine species. Mech. Ageing Dev., 27, 239-247.

Subba Rao, K. (1973) Acid deoxyribonuclease activity in developing human foetal brain. Life Sci., 12, 89-96.

Sutherland, B.M., Harber, L.C. and Kochevar, I.E. (1980) Pyrimidine dimer formation and repair in human skin. Cancer Res., 40, 3181-3185.

Sutherland, B.M. and Shih, A.G. (1983) Quantitation of pyrimidine dimer contents of nonradioactive deoxyribonucleic acid by electrophoresis in alkaline agarose gels. Biochemistry., 22, 745-749.

Swaab, D.F., Fliers, E., Miran, M., Van Gool, W. and Van Haren, F. (1988) Aging of the brain and Alzheimer's disease. Prog. Brain Res. Vol. 70, Elsevier, Amsterdam.

Taichman, L.B. and Setlow, R.B. (1979) Repair of ultraviolet light damage to the DNA of cultured human epidermal keratinocytes and fibroblasts. J. Invest. Dermatol., 73, 217-219.

Takebe, H., Yagi, T. and Satoh, Y. (1983) Cancer prone hereditary diseases in relation to DNA repair. in: 13th International Cancer Congress, Part B, Biology of Cancer (1), Liss, New York, pp. 267-275.

Tates, A.D., Bernini, L.F., Natarajan, A.T., Ploem, J.S., Verwoerd, N.P., Cole, J., Green, M.H.L., Arlett, C.F. and Norris, P.N. (1989) Detection of somatic mutants in man: HPRT mutations in lymphocytes and hemoglobin mutations in erythrocytes. Mutation Res., 213, 73-82.

Téoule, R. (1987) Radiation-induced DNA damage and its repair. Int. J. Radiat. Biol., 51, 573-589.

Terry, R.D. (1983) Cortical morphometry in Alheimer's disease. in: Katzman, R. (Eds.), Biological Aspects of Alzheimer's Disease, Cold Spring Harber Laboratory, New York, pp 95-98.

Tice, R.R. and Setlow, R.B. (1985) DNA repair and replication in aging organisms and cells. in: Finch, C.E. and Schneider, E.L. (Eds.), Handbook of the Biology of Aging, 2nd ed., Van Nostrand Reinhold Company, New York, pp. 173-224.

Tonegawa, S. (1980) Somatic generation of antibody diversity. Nature, 302, 575-581

Tong, C., Laspia, M.F., Telang, S. and Williams, G.M. (1981) The use of adult liver cultures in the detection of the genotoxicity of various polycyclic aromatic hydrocarbons. Environ. Mutagenesis, 3, 477-478.

Trainor, K.J., Wigmore, D.J., Chrysostomu, A., Dempsey, J.L., Seshadri, R. and Morley, A.A. (1984) Mutation frequency in human lymphocytes increases with age. Mech. Ageing Dev., 27, 83-86.

Turturro, A. and Hart, R.W. (1984) DNA repair mechanisms in aging. in: Scarpelli, D. and Mikagi, G. (Eds.), Comparative pathobiology of major age-related diseases: Current status and research frontiers, Liss, New York, pp. 19-45.

Ueda, K. (1985) ADP-ribosylation. Ann. Rev. Biochem., 54, 73-100

Uitterlinden, A.G., Slagboom, P., Knook, D.L. and Vijg, J. (1989) Two-dimensional DNA fingerprinting of human individuals. Proc. Natl. Acad. Sci. USA., 86, 2742-2746.

UNSCEAR report (1988) Sources, Effects and Risks of Ionizing Radiation, UNSCEAR, New York, pp. 49-135.

Vaca, C.E., Wilhelm, J. and Harms-Ringdahl, M. (1988) Interaction of lipid peroxidation products with DNA. A review. Mutation Res., 195, 137-149.

Van Bezooijen, C.F.A. (1984) Influence of age-related changes in rodent liver morphology and physiology on drug metabolism - A review. Mech. Ageing Dev., 25, 1-22.

Van Bezooijen, C.F.A. (1986) Changes in pharmacokinetics with age in man. A review. in: Van Bezooijen, C.F.A., Miglio, F. and Knook, D.L. (Eds.), Liver, Drugs and Aging, Eurage, Rijswijk, pp. 25-32.

Van Bezooijen, C.F.A., Bukvic, S., Sleyster, E.Ch. and Knook, D.L. (1984) Bromsulfophthalein storage capacity of rat hepatocytes separated into ploidy classes by centrifugal elutriation. in: Van Bezooijen, C.F.A. (Eds.), Pharmacological, Morphological and Physiological Aspects of Liver Aging, Pasmans, 's-Gravenhage, pp. 115-120.

Van der Schans, G.P., Centen, H.B. and Lohman, P.H.M. (1982) DNA lesions induced by ionizing radiation. in: Natarajan, A.T., Obe, G. and Altmann, H. (Eds.), Progress in Mutation Research, Vol 4, Elsevier Biochemical, New York, pp. 285-299.

Van der Schans, G.P., Vos, O., Roos-Verheij, W.S.D. and Lohman, P.H.M. (1986) The influence of oxygen on the induction of radiation damage in DNA in mammalian cells after sensitization by intracellular glutathione depletion. Int. J. Radiat. Biol., 50, 453-465.

Van Zwieten, M.J. (1984) The rat as animal model in breast cancer research. Thesis, Martinus Nijhoff Publischers, Boston.

Vijayalaxmi and Evans, H.J. (1984) Measurement of spontaneous and X-irradiation-induced 6-thioguanine-resistent human blood lymphocytes using a T-cell cloning technique. Mutation Res., 125, 87-94.

Vijg, J. (1987) DNA repair and the aging process. Thesis, Pasmans, 's Gravenhage.

Vijg, J. and Knook, D.L. (1987) DNA repair in relation to the aging process. J. Am. Ger. Soc., 35, 532-541.

Vijg, J. and Uitterlinden, A.G. (1987) A search for DNA alterations in the aging mammalian genome: an experimental strategy. Mech. Ageing Dev., 41, 47-63.

Vijg, J., Mullaart, E., Van der Schans, G.P., Lohman, P.H.M. and Knook, D.L. (1984) Kinetics of ultraviolet-induced DNA excision repair in rat and human fibroblasts. Mutation Res., 132, 129-138.

Vijg, J., Mullaart, E., Lohman, P.H.M. and Knook, D.L. (1985) UV- induced unscheduled DNA synthesis in fibroblasts of aging inbred rats. Mutation Res., 146, 197-204.

Vijg, J., Mullaart, E., Berends, F., Lohman, P.H.M. and Knook, D.L. (1986a) UV-induced DNA excision repair in rat fibroblasts during immortalization and terminal differentiation in vitro. Exp. Cell Res., 167, 517-530.

Vijg, J., Mullaart, E., Roza, L., Baan, R.A. and Lohman, P.H.M. (1986b) Immunochemical detection of DNA in alkaline sucrose gradient fractions. J. Imm. Meth., 91, 53-58.

Vijg, J., Roza, L., Mullaart, E. and Berends, F. (1987) DNA repair in relation to skin aging, Giornale Italiano di Dermatologia Chirurgica, 2, 300-311.

Visser, A. and Westra, J.G. (1981) Partial persistency of 2-aminofluorene and N-acetyl-2-aminofluorene in rat liver DNA. Carcinogenesis, 2, 737-740.

Vos, O. and Roos-Verhey, W.S.D. (1988) Radioprotection by glutathione esters and cysteamine in normal and glutathione-depleted mammalian cells. Int. J. Radiat. Biol., 53, 273-281.

Wade, M.H. and Lohman, P.H.M. (1980) DNA repair and survival in UV-irradiated chicken embryo fibroblasts. Mutation Res., 70, 83-93.

Waksvik, H.P., Magnus, P. and Berg, K. (1981) Effects of age, sex and genes on sister chromatid exchange. Clin. Genet., 20, 449-454.

Walker, A.P. and Bachelard, H.S. (1988) Studies on DNA damage and repair in the mammalian brain. J. Neurochem., 51, 1394-1399.

Webster, H.L. and Harrison, D.D. (1969) Enzymic activities during the transformation of crypt to columnar intestinal cells. Lipids, 19, 423-435.

Wheeler, K.T. and Lett, J.T. (1974) On the possibility that DNA repair is related to age in non-dividing cells. Proc. Natl. Acad. Sci. USA., 71, 1862-1865.

Wheeler, K.T. and Wierowski, J.V. (1983) DNA repair kinetics in irradiated undifferentiated and terminally differentiated cells. Radiat. Environ. Biophys., 22, 3-20.

Wilson, V.L., Smith, R.A., Ma, S. and Cutler, R.G. (1987) Genomic 5-methyldeoxycytidine decreases with age. J. Biol. Chem., 262, 9951-9984.

Williams, G.M. (1977) Detection of chemical carcinogens by unscheduled DNA synthesis in rat primary cultures. Cancer Res., 37, 1845-1851.

Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. (1984) Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathology, 142, 67-77.

Yagi, T. (1982) DNA repair ability of cultured cells derived from mouse embryos in comparison with human cells. Mutation Res., 96, 89-98.

Yamamoto, O., Fuji, I., Yoshida, T., Cox, A.B. and Lett, J.T. (1988) Age dependency of base modification in rabbit liver DNA. J. Gerontol., 43, B132-136.

Yu, F-l. (1983) Preferential binding of aflatoxin B1 to the transcriptionally active regions of rat liver nucleolar chromatin in vivo and in vitro. Carcinogenesis, 4, 889-893.

Zelle, B. and Lohman, P.H.M. (1979) Repair of UV-endonuclease susceptible sites in 7 complementation groups of xeroderma pigmentosum A through G. Mutation Res., 62, 363-368.

LIST OF PUBLICATIONS[1]

Verheijen, J.H., Mullaart, E., Chang, G.T.G., Kluft, C. and Wijngaards, G. (1982) A simple, sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. Thromb. Haemostasis, 48, 266-269.

Vijg, J., Mullaart, E., Van der Schans, G.P., Lohman, P.H.M. and Knook, D.L. (1984) Kinetics of ultraviolet-induced DNA excision repair in rat and human fibroblasts. Mutation Res., 132, 129-138.

Vijg, J., Mullaart, E., Lohman, P.H.M. and Knook, D.L. (1985) UV-induced unscheduled DNA synthesis in fibroblasts of aging inbred rats. Mutation Res., 146, 197-204.

Vijg, J., Mullaart, E., Berends, F., Lohman, P.H.M. and Knook, D.L. (1986) UV-induced DNA excision repair in rat fibroblasts during immortalization and terminal differentiation in vitro. Exp. Cell Res., 167, 517-530.

Vijg, J., Mullaart, E., Roza, L., Baan, R.A. and Lohman, P.H.M. (1986) Immunochemical detection of DNA in alkaline sucrose gradient fractions. J. Imm. Meth., 91, 53-58.

Mullaart, E., Lohman, P.H.M. and Vijg, J. (1988a) Differences in pyrimidine dimer removal between rat skin cells in vitro and in vivo. J. Invest. Dermatol., 90, 346-349.

Mullaart, E., Boerrigter, M.E.T.I., Brouwer,A., Berends, F. and Vijg, J. (1988b) Age-dependent accumulation of DNA alkali-labile sites in DNA of post-mitotic but not in that of mitotic rat liver cells. Mech. Ageing Develop., 45, 41-49.

Mullaart, E., Buytenhek, M., Brouwer, A., Lohman, P.H.M. and Vijg, J. (1989c) Genotoxic effects of intragastrically administered benzo(a)pyrene in rat liver and intestinal cells. Carcinogenesis, 10, 393-395.

Mullaart, E., Roza, L., Lohman, P.H.M. and Vijg, J. (1989a) The removal of UV-induced pyrimidine dimers in rat skin cells in vitro and in vivo in relation to aging. Mech. Aging Develop., 47, 253-264.

[1] Publications in reviewed journals.

Mullaart, E., Boerrigter, M.E.T.I., Lohman, P.H.M. and Vijg, J. (1989b) Age-related induction and disappearance of carcinogen-DNA-adducts in livers of rats exposed to low levels of 2-acetylaminofluorene. Chem. Biol. Interact., 69, 373-384.

Boerrigter, M.E.T.I., Mullaart, E., Van der Schans, G.P. and Vijg, J. (1989) Quiescent human peripheral blood lymphocytes do not contain a sizable amount of preexistent DNA single-strand breaks. Exp. Cell Res., 180, 569-573.

Gille, J.J.P., Mullaart, E., Vijg, J., Leyva, A.L., Arwert, F. and Joenje, H. (1989) Chromosomal instability in an oxygen-tolerant variant of chinese hamster ovary cells. Mutation Res., 219, 9-16.

Gille, J.J.P., Van Berkel, C.G.M., Mullaart, E., Vijg, J. and Joenje, H. (1989) Effects of lethal exposure to hyperoxia and to hydrogen peroxide on NAD(H) and ATP pools in Chinese hamster ovary cells. Mutation Res. in press

Mullaart, E., Boerrigter, M.E.T.I., Boer, G.J. and Vijg, J. (1989d) The level of spontaneous DNA breaks in rat brain during development and aging. Submitted for publication.

Mullaart, E., Boerrigter, M.E.T.I., Ravid, R., Swaab, D.F. and Vijg, J. (1989e) Increased levels of DNA breaks in cerebral cortex of Alzheimer's disease patients. Submitted for publication.

Boerrigter, M.E.T.I., Mullaart, E. and Vijg, J. (1989b) Induction and repair of DNA strand breaks in human lymphocytes exposed to N-ethyl-N-nitrosourea. Submitted for publication.

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