MODULATION OF GROWTH CONTROL MECHANISMS CRITICAL TO ATHEROGENESIS

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Proefschrift

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in de landbouw- en milieuwetenschappen

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STELLINGEN

1. De verstoring van intercellulaire communicatie via zogenaamde 'gap junctions' is een sleutelproces in de atherogenese.
   - *Dit proefschrift.*

2. In de atherogenese zijn vooral geoxideerde lipoproteinen van belang en niet het cholesterol als zodanig.
   - *R.A. Riemersma, D.A. Wood, C.C.A. MacIntyre, R.A. Elton, K.F. Gey, M.F. Oliver, Lancet 337, 1-5, 1991.*
   - *D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo, J.L. Witztum, New Engl. J. Med. 320, 915-924, 1989.*
   - *Dit proefschrift.*

3. De 'response-to-injury' theorie en de 'monoclonale' theorie sluiten elkaar niet wederzijds uit.
   - *Dit proefschrift.*

4. Aan het ontstaan van atherosclerose en kanker onder invloed van chemische factoren kunnen vergelijkbare mechanismen ten grondslag liggen.
   - *M.W. Majesky, M.A. Reidy, E.P. Benditt, M.R. Juchau, Proc. Natl. Acad. Sci. 82, 3450-3454, 1985.*
   - *Dit proefschrift.*

5. De hypothese dat cholesterol-verlagende behandelingen kunnen leiden tot toename van het aantal zelfmoorden en andere ongevallen bevat onvoldoende argumenten op grond waarvan een oorzakelijk verband plausibel wordt gemaakt en is derhalve speculatief.
   - *M.F. Muldoon, S.B. Manuck, K.A. Matthews, Br. Med. J. 301, 309-314, 1990.*
   - *H. Engelberg, Lancet 339, 727-729, 1992.*

6. Ondanks het feit dat het fenomeen apoptose al 20 jaar bekend is wordt hiermee in de toxicologie te weinig rekening gehouden.

7. In het kader van de interdisciplinaire samenwerking dreigt de toxicologie zijn relatie met de biomoleculaire wetenschappen te verliezen.

8. De veiligheidsfactor, welke toegepast wordt bij het vaststellen van de 'acceptable daily intake', zou afhankelijk moeten worden van de beschikbare gegevens betreffende inter- en intraspecies variatie in toxicokinetiek en toxicodynamiek van deze stoffen.
- A.G. Renwick, Food Additives and Contaminants 8, 135-150, 1991.

9. Bij de beoordeling van onderzoeksgegevens wordt het belang van de reproduceerbaarheid in vergelijking met de statistische significantie in het algemeen ondergewaardeerd.

10. Het verkrijgen van gelijke rechten voor kamerhuurder en -verhuurder draagt bij tot een oplossing van de kamernood.

11. De wet van Archimedes kan als volgt gemodificeerd worden: een juiste combinatie van opwaartse arbeidsdruk en neerwaartse diepzee waterdruk is de drijfveer van het individu.

Stellingen behorende bij het proefschrift "Modulation of growth control mechanisms critical to atherogenesis". Renate M.L. Zwijsen, Wageningen, 13 november 1992.

Ik hoop dat de zee, die al duizenden jaren de mens wijsheid tracht te leren, hem wederom zal inspireren, nu tot gedachten en daden die het evenwicht en het leven op aarde zullen handhaven.

Jacques-Yves Cousteau

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CONTENTS

ABBREVIATIONS

BaP	benzo[a]pyrene
BHT	butylated hydroxytoluene
BSA	bovine serum albumine
CSC	cigarette smoke condensate
DMSO	dimethylsulfoxide
EMEM	Eagle's minimal essential medium
FCS	fetal calf serum
FGF	fibroblast growth factor
HBGF	heparin binding growth factor
HDL	high density lipoproteins
IGF-1	insuline-like growth factor 1
Il-1	interleukine 1
LDH	lactate dehydrogenase
LDL	low density lipoproteins
LPDS	lipoprotein deficient serum
MCA	3-methylcholantrene
MDA	malondialdehyde
PDGF	platelet-derived growth factor
SSC	standard saline citrate
ssDNA	salmon sperm DNA
TBARS	thiobarbituric acid reactive substances
TGF-ß	transforming growth factor ß
TNF	tumor necrosis factor
TPA	12-O-tetradecanoyl-phorbol-13-acetate

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Atherosclerosis and its complications, such as myocardial infarction, stroke and peripheral vascular disease, are the major causes of morbidity and mortality in the Western World. The disease is characterized by an abnormal proliferation of smooth muscle cells in the intima of the artery vessel wall. Epidemiological studies show a strong association between atherosclerosis and a variety of environmental factors as cigarette smoke and agents causing hypertension (Hopkins and Williams, 1981; Seidel and Cremer, 1986). However, direct evidence linking these factors to atherosclerosis in a causal manner is lacking. Therefore, research of (atherogen-induced) molecular mechanisms critical to atherogenesis is needed.

The assumption that certain chemicals could play a role in plaque formation comparable to the role carcinogens play in tumor formation is of toxicological interest. A parallel between carcinogenesis and atherogenesis is demonstrated by the facts that carcinogenic compounds, oncogenic viruses and radiation are able to induce lesions in a number of animal species, which shows similarities with atherosclerotic lesions found in humans (Albert et al., 1977; Bond et al., 1981; Gold, 1961; Fabricant et al., 1978; Majesky et al., 1985).

If it is true that certain compounds with mutagenic potencies could also play an important role in human atherosclerosis, it would be of paramount importance to study the role of chemicals in the etiology of atherosclerosis in more detail.

ETIOLOGY OF ATHEROSCLEROSIS

A parallel between carcinogenesis and atherogenesis was brought forward by Benditt and Benditt (1973) and presented as the monoclonal hypothesis. Based on comparative immunochemical examination of glucose-6-phosphate dehydrogenase isoenzymes isolated from healthy arterial walls and atherosclerotic lesions, evidence was obtained for a monoclonal origin of atherosclerotic lesions. It was supposed that every lesion is in fact a benign tumor that has developed from one single smooth muscle cell, which has mutated through the action of chemical substances, ionizing radiation or viruses. Arguments in

favor of this hypothesis are that tumor initiators like chemical mutagens and promutagens (Albert et al., 1977; Bond et al., 1981), certain forms of radiation (Gold, 1961) and oncogenic viruses (Fabricant et al., 1978) can induce atherosclerotic lesions in laboratory animals. Furthermore, the polycyclic aromatic hydrocarbon carcinogen 7,12-dimethylbenzo[a]anthracene, administered as an "initiator" followed by treatments with the "promoter" methoxane yielded aortic plaques in cockerels (Majesky et al., 1985).

Another polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP), present in e.g. cigarette smoke, can be converted by aortic tissue to diol epoxides (Juchau et al., 1976; Serabjit-Singh et al., 1985; Bond et al., 1979; Majesky et al., 1983). Moreover, the "in vitro" DNA-damaging action of BaP metabolites was demonstrated using three test systems with different genetic endpoints used: sister-chromatid exchange, gene mutation at the hypoxanthine guanine phosphoribosyl transferase locus and unscheduled DNA synthesis (Zwijsen et al., 1990). These three test systems were also used to compare the metabolic activation capacity towards BaP of aorta smooth muscle cells of different species (rat, rabbit) and locations (thoracic and abdominal) in order to investigate a relation between the formation of mutagenic metabolites and the susceptibility to atherosclerosis. However, such a correlation was not observed (Zwijsen et al, 1990). As smooth muscle cells appeared to have a low metabolic capacity towards BaP the conclusion could be drawn that differences in susceptibility between species can not be explained solely on the basis of differences in biotransformation capacity, other factors are probably more important.

Furthermore, studies by Penn et al. (1986) and Ahmed et al. (1990) provided additional evidence for the predictions of the monoclonal hypothesis by demonstrating that human coronary artery plaque DNA contained transforming potential and that mouse fibroblasts transfected with human plaque DNA give rise to tumors after subcutaneous injection into nude mouse. However, Yew et al. (1989) using the same material and methods, were not able to demonstrate transforming genes in atherosclerotic plaques.

Despite the available support the monoclonal theory has not been widely accepted, which is partly due to the contradictory results. The generally accepted view of plaque development still is to regard smooth muscle cell proliferation as a reactive process, which occurs in response to stimuli such as injury or inflammation. According to that hypothesis smooth muscle cells involved in lesion formation do not genetically differ from the bulk of normal arterial smooth muscle cells.

The response-to-injury hypothesis of Ross (1976/1981) is based on the observations that endothelium denudation of arteries by a balloon embolectomy catheter produces a prominent platelet carpet on the denuded surface, followed by intimal proliferation, and that antiplatelet serum inhibits the smooth muscle proliferative response (Friedman et al., 1977). It was proposed that smooth muscle cells in the wall normally exist in a quiescent state, but when the endothelium is injured, adhesion and aggregation of platelets to the damaged endothelium resulted in a release of factor(s), as platelet-derived growth factor (PDGF), that stimulate medial smooth muscle cells to migrate into the intima and to proliferate there.

Another theory, the lipid hypothesis, states that circulating cholesterol is central to the pathogenic process, causing smooth muscle cell proliferation. Cholesterol bound to low density lipoproteins (LDL) is capable of damaging endothelium and of infiltrating into the tunica intima (Hollander et al., 1979). There, the cholesterol concentration of the cell is regulated by receptors which bind LDL-cholesterol complexes and then make the latter available to the cell for hydrolysis and esterification. The free cholesterol is partly used for membrane synthesis but also by means of a feedback mechanism suppresses the endogenous synthesis of cholesterol and the LDL-receptors. The accumulation of lipids in the proliferating cells would be due to a defective regulation mechanism, to an inadequate catabolism or to formation of insoluble cholesterol esters (Brown, 1981).

All theories explaining atherogenesis, including the Benditt theory, are based on the concept of a defect growth control mechanism for smooth muscle. As impaired growth control is a key process in atherogenesis as well as in carcinogenesis and keeping in mind several parallels between the two diseases absences of growth control mechanism in tumors and atherosclerotic lesions this unifying aspect of the two diseases will be discussed in more detail in the following paragraphs.

ABSENCE OF GROWTH CONTROL IN TUMORS AND PLAQUES

Tumorigenesis

Tumor development and progression may be viewed as a gradual emancipation of a clone of somatic cells from the complex controls that regulate its growth (Klein and Klein,

1985). All neoplastic cells have lost some measure of growth control and the cellular elements involved include growth factors, growth factor receptors and oncogenes (Bradshaw, 1986). Genes with either a normal or pathological function may influence the steps of tumor development in a positive or negative way. Such genes may be provisionally classified as oncogenes (genes that cause cancer), emerogenes (also called antioncogenes or tumor suppressor genes), and modulator genes (which can influence important but secondary malignant properties such as invasiveness, metatastic propensity, or the ability to generate an immune response). The discovery of oncogenes stems from the search for tumor viruses, stimulated by earlier successfull developments in microbiology and the epidemiology of infectious diseases. Afterwards, it was discovered that most of these genes are important cellular genes involved in the control of cell division. Undue activation of the cellular oncogenes by point mutation, retroviral insertion, chromosomal translocation or gene amplification can contribute to tumor development and progression in many systems (Bishop, 1987). Several of the currently known oncogenes can block specific steps in the maturation progress. Constituvely activated growth factors may inhibit maturation by urging their target cell to proliferate. Truncated growth factor receptors or faulty signal transducers may achieve a similar effect by emitting a continuous "go" signal in the absence of external stimulation. DNA-binding proteins, like myc or myb, may block maturation by interfering with the condensation of chromatin which is the hallmark of terminal differentiation in many cells.

Also the category of genes described as antioncogenes, tumor suppressor genes or emerogenes, respectively, can antagonize tumorigenic behavior at various levels. For example, the constitutive activation of a 'growth factor oncogene' may be canceled by the loss or dysfunction of the corresponding receptor, by a roadblock elsewhere within the complex pathway of signal transmission, as well as by changes in the responding target. In addition, oncogene-induced blocks to cell maturation may be overcome by strong inducers or circumvented by the use of alternative pathways (Klein, 1987). So, the regulation of cell growth is operated by a balanced action of growth factors, growth factor receptors and products of oncogenes, emerogenes and modulator genes.

Growth control mechanisms in arterial smooth muscle cells.

In arterial vessels in general three coats surrounding the lumen are visible: the internal tunica intima, the intermediate tunica media and the external tunica adventitia. The tunicae have different functions and are each associated with different major cell types. The intima, responsible for exchange with the blood is characterized by the presence of endothelial cells, the media which is involved in the control of the diameter of medium and small vessels and the mechanical integrity of large ones, contains large numbers of smooth muscle cells and the adventitia, responsible for nourishing and protecting the vessels, contains connective tissue cells, nerve cells and smaller blood vessels (vasa privata). The smooth muscle cell is the main cell type involved in the pathogenesis of atherosclerosis, which proliferate and migrate from the arterial media into the subendothelial space (Haust et al., 1960; Ross and Glomset, 1976; Moss and Benditt, 1970; Schwartz et al., 1986). However, interactions between smooth muscle cells and neighboring tissues, especially endothelial cells, platelets and monocyte/macrophages play an important role in the biology of the vessel wall and possibly participate in the pathogenesis of atherosclerosis. Three main mechanisms can explain smooth muscle growth control:

a. Smooth muscle growth may be controlled by paracrine production of growth stimulating or inhibiting factors released from blood cells and endothelial cells (Ross, 1990).
b. Smooth muscle growth may be controlled by autocrine production of growth stimulating or inhibiting factors (Wilcox et al., 1988).
c. Smooth muscle growth may be controlled by cell-cell communication (this study).

Paracrine production of growth factors for smooth muscle.

A large number of molecules are mitogenic for quiescent smooth muscle cells in culture. Platelets release platelet-derived growth factor (PDGF), insuline-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and transforming growth factor ß (TGF-ß), growth factors which are able to assist smooth muscle replication (Clemmons et al., 1983; Oka and Orth, 1983; Associan et al. 1984; Tucker et al, 1984).

Besides platelets, endothelial cells and activated macrophages also have the capacity to express the genes for growth factors, e.g. PDGF A and B as well as for interleukin 1 (Il-

1) (Martinet et al., 1986; Starksen et al., 1987; Bevilacqua et al., 1984). In addition, endothelial cells contain mitogenic heparin binding growth factors (HBGF), i.e. acidic and basic fibroblast growth factors (FGF) (Gospodarowicz et al., 1988). Lysates of endothelial cells containing FGF were able to stimulate smooth muscle cell replication in absence of growth factors (Gajdusek and Schwartz, 1984; Stavnow and Berg, 1987).
Smooth muscle proliferation could, on the other hand, be theoretically ascribed to the loss of an inhibitory molecule present in the vascular wall (Castellot et al., 1981; Reilly et al., 1987). Such molecules have been described, including heparans and proteoglycans, which are most prominent in quiescent cultures (Fritze et al., 1985). Both vascular endothelial and smooth muscle cells synthesize heparan sulphate molecules that can inhibit smooth muscle cell growth. These growth inhibitors can be inactivated by release of specific enzymes. Castellot et al. (1982) proposed that following endothelial denudation or injury, platelets are deposited on the subendothelium releasing heparitinase, platelet factor 4 and PDGF. The heparitinase cleaves heparin-like substances on the endothelium and smooth muscle into fragments which diffuse away, with additional inactivation of the substance by platelet factor 4 (Handin and Cohen, 1976). Thus it could be possible that endothelium injury allows smooth muscle growth by breakdown of heparan sulphate.

Autocrine production of growth factors.

Intimal smooth muscle cell proliferation can occur without the presence of platelets in the vascular lumen (Guyton and Karnovsky, 1979). So, the extent of cell replication is not dependent on the presence of platelet released factors and it is at least possible for smooth muscle cells to initiate DNA synthesis without interacting with platelets or other vessel wall cells (Fingerle and Kraft, 1987). In addition, arterial smooth muscle cells produce growth promoting substances, as PDGF-A, IGF-1, TGF ß and FGF, themselves (Clemmons and Van Wyk, 1985; Majesky et al., 1988; Gospodarowicz et al., 1988; Winkles et al., 1987). The hypothesis that an autocrine growth control mechanism is involved in atherogenesis is supported by the findings that plaque DNA contains an active transforming gene(s) (Penn et al., 1986; Ahmed et al., 1990). Further support can be derived from data demonstrating that PDGF-A expression is increased in human intimal lesion cells inside human atherosclerotic lesions and in cultured plaque cells (Wilcox et al., 1988; Libby et al., 1988). Since human arterial smooth muscle cells can both produce and

respond to PDGF-A the increase of PDGF-A transcripts in intimal cells suggests an important role of this gene in atherogenesis.

Intercellular communication.

Gap junctions, which are composed of arrays of intramembraneous channels, act as specialized contact zones between neighboring cells and allow for the passage of ions and molecules of up to 1000 daltons (Simpson et al., 1977). Intercellular communication by gap junctions is considered to play an important role in maintaining and controlling cell growth, cell differentiation and homeostasis. It exerts an inhibitive effect on tumor cell precursors that contain activated oncogenes. Reduction of cell-cell communication by modulating the gap junctions could result in a clonal growth, ignoring neigboring normal cells. Indeed, evidence that tumor promoters can inhibit intercellular communication was first obtained in 1979 by Yotti et al. and Fitzgerald & Murray. Afterwards, a great deal of evidence shows that the blockage of gapjunctional communication might be an essential process in the promotion stage of carcinogenesis (reviewed by Yamasaki, 1990). Many types of promoting agents inhibit intercellular communication, which correlated well with their *in vivo* tumor promoting capacity (Trosko et al., 1982). Even several oncogene products such as from the *src* and *ras* genes, growth factors and viruses have shown to be associated with a decrease in cell-cell communication (Azarnia and Loewenstein, 1984; Chang et al., 1985; Madhukar et al., 1989). In addition, Land (1986) have shown that both *myc*- and *ras*- transformed rat fibroblasts can be suppressed by surrounding normal cells. So, blockage of junctional communication is now believed to be involved in the promotional phase of carcinogenesis. Since this control system is absent from tumors as well as plaques disturbance of cell-cell communication also could be involved in atherogenesis.

AIM OF THE STUDY

The main objective of this study is to investigate the modulation of growth control mechanisms critical to atherogenesis. As most experimental work has been performed in the framework of paracrine growth regulation we decided to focus our study on autocrine

growth regulation and gap junctional intercellular communication as a novel mechanism relevant to atherogenesis. The long term objective of the programme is to support the development of toxicological research models enabling the study of effects of chemicals on these growth control processes. In this study we tested the influence of some known atherogens on these growth control mechanisms. Therefore, we intended to use material of human origin as much as possible to enhance their relevance with regard to the human species.

The atherogen-induced disturbances of growth control mechanism by inhibition of gap junctional intercellular communication are described in chapters 2,3 and 4. Chapters 5 and 6 describe a transforming potential of atherosclerotic lesion DNA and low density lipoproteins, respectively. Chapters 5 and 7 deal with the possible involvement of PDGF-A in atherogenesis. The summary and the conclusions are presented in chapters 8 and 9.

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CHAPTER 2

INHIBITION OF INTERCELLULAR COMMUNICATION IN SMOOTH MUSCLE CELLS OF HUMAN AND RATS BY LOW DENSITY LIPOPROTEINS, CIGARETTE SMOKE CONDENSATE AND TPA

Inhibition by tumor promoting chemicals of intercellular communication via gap junctions may be important in carcinogenesis. In order to investigate the possible role of gap junctional intercellular communication in atherogenesis, we examined the effect of known inhibitors of intercellular communication, 12-O-tetradecanoylphorbol-13-acetate (TPA) and Cigarette Smoke Condensate (CSC), and Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL) on cellular communication in smooth muscle cells of human and rat by the microinjection-dye transfer technique.

When Lucifer Yellow CH solution is injected into a cell, the average number of human and rat smooth muscle cells that become fluorescent is about 22 and 6, respectively. The tumor promoter (TPA) almost completely blocked gap junctional communication between smooth muscle cells at 100 ng/ml after 4 h exposure. LDL and CSC were able to inhibit intercellular communication in human and rat cells in a dose-dependent manner up to 60%. LDL-pretreatment of human smooth muscle cells did not effect inhibition of intercellular communication, which suggests that this effect is mainly non-receptor mediated. HDL did not influence junctional communication. The results indicate that inhibition of intercellular communication may also contribute to the pathogenesis of atherosclerotic lesions, such as plaque.

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Renate M.L. Zwijsen, Laura H.J. de Haan, Jeanine S. Oosting, Hadewijch L.M. Pekelharing and Jan H. Koeman (1990) Inhibition of intercellular communication in smooth muscle cells of humans and rats by low density lipoprotein, cigarette smoke condensate and TPA. Atherosclerosis 85, 71-80.

INTRODUCTION

Intercellular communication by gap junctions is considered an important cellular mechanism for regulating growth and differentiation (Loewenstein, 1979; Bennet and Spray, 1985). Gap junctions are hydrophilic channels employed for the exchange of important signal ions and molecules between cells. Inhibition of intercellular communication may be the key mechanism by which certain tumor promoting chemicals contribute to the etiology of chemical carcinogenesis (Loewenstein, 1979). Tumor promoting agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and cigarette smoke condensate (CSC), have been shown to inhibit intercellular communication in various cell types (Yotti et al., 1979a/b; Murray and Fitzgerald, 1979; Mosser and Bols, 1982; Enomoto and Yamasaki, 1985; Newbold and Amos, 1981; Ruch et al., 1987; Rutten et al., 1988; Hartman and Rosen, 1983).

The fibrous plaque is regarded as the most characteristic vascular lesion of atherosclerosis. Plaques represent abnormal proliferations of smooth muscle cells, which seem to have a monoclonal character for isoenzymes of glucose-6-phosphatedehydrogenase (G-6-PD) (Benditt and Benditt, 1973; Pearson et al., 1975). The finding of a high frequency of monoclonality for G-6-PD in atherosclerotic plaques led to the suggestion that the plaque is a primary benign neoplasm (Benditt and Benditt, 1973). Atherosclerotic lesions can be ultimately formed from single mutated smooth muscle cells in the intima of large arteries. After initiation of a preneoplastic lesion followed by a promotion stage, the smooth muscle cells proliferate and ultimately form a tumor: the plaque.

Several investigations provide support for the monoclonal hypothesis of Benditt (Paigen et al., 1985; Albert et al., 1977; Penn et al., 1986). Carcinogens such as 3-methylcholantrene, benzo(a)pyrene and dimethylbenzantracene induce atherosclerotic lesions in laboratory animals (Paigen et al., 1985; Albert et al., 1977). In addition, Penn and colleagues (Penn et al., 1986) provide evidence for the existence of an active human oncogene in plaques by transformation of cultured mouse fibroblast cells with transfected DNA from human plaques. This would imply that mutations lead to activation of oncogenes in the cells that make up the atherosclerotic plaque, a process demonstrated previously only in cancer cells.

The aim of the present investigation was to study the effect of lipoproteins and cigarette

smoke condensate (CSC) on gap junctional communication in smooth muscle cells of human and rat. Intercellular communication was measured by a dye transfer assay using arterial smooth muscle cells of human and rat. The following aspects were studied: (a) the effect of known inhibitors of intercellular communication, the tumorpromotors TPA and CSC, on communication in human and rat smooth muscle cells; (b) the effect of LDL and HDL on intercellular communication in this model system.

MATERIALS AND METHODS

Reagents

LDL, HDL, 12-O-tetradecanoylphorbol-13-acetate (TPA) and Lucifer Yellow CH were purchased from Sigma Chemical Co (U.S.A.). Cigarette-smoke condensate (CSC) was a gift from ITV-TNO, Zeist, The Netherlands. CSC and TPA were dissolved in dimethyl-sulfoxide (DMSO). DMSO and Triton X-100 were obtained from E. Merck EG (F.R.G.). Sodium pyruvate was obtained from BDH Chemicals Ltd (U.K.) and NADH from Boehringer (F.R.G.).

LDL purity

The LDL purity was controlled on breakdown and oxidation products by means of agarose gelelectrophoresis and the thiobarbituric acid reactive substances (TBARS) assay as previously described (Steinbrecher et al., 1984). The amount of malondialdehyde (MDA) equivalent was determined using freshly diluted 1,1,3,3-tetramethoxypropane (Aldrich, F.R.G.) as a standard. The degree of LDL oxidation was expressed in nanomoles of MDA equivalent per milligram of LDL-cholesterol. No degradation or oxidation of control LDL preparations were observed. This was shown by the small band of lipid staining (fat red 7B) after agarose gelelectrophoresis and the TBARS values for control LDL preparations, which were always lower than 0.2 nmol MDA/mg LDL-cholesterol.

Cell culture

Arterial smooth muscle cells were obtained from arteries of human umbilical cord and abdominal aorta of Wistar rats (6-8 months) by an explant technique (Ross, 1971). In brief, arteries were isolated aseptically, unwanted tissue was dissected off and the tissue was chopped in pieces of 1 mm. The pieces were washed 3-5 times with PBS (Oxoid Limited, UK), placed on glass coverslips in 6-well dishes (Costar, U.S.A) and incubated for 10 days in Eagle's Minimal Essential Medium (EMEM; Flow Laboratories, UK) supplemented with 5 % Fetal Calf Serum (FCS, Gibco, UK) and 5 % human serum (Sigma Chemicals, St. Louis, MO) or 10 % FCS (human and rat cells, respectively), gentamycin (50 μg/ml; Gibco) and amphotericin B (1.5 μg/ml; Gibco) in a 37 °C, 5 % CO_2 humidified air environment.

After 10-15 days, if sufficient periferal growth had occurred, cells were subcultured in flasks (Costar) by trypsination. Distinction between smooth muscle, fibroblasts and endothelial cells was made by means of fluoresceinated antibodies against smooth muscle actin (Chamley et al., 1977) and the growth pattern of smooth muscle cells in culture (hills and valleys). The smooth muscle cell cultures which were used in our studies were free of fibroblasts and endothelial cells.

Measurement of intercellular communication by means of fluorescent dye transfer

Smooth muscle cells (passage 2-3) were plated at a concentration of 2 x 10^5 cells per well (3.5 cm diameter) on a glass cover slip (1.8 x 1.8 cm) and cultured for two days in EMEM supplemented with FCS (5 %) and human serum (5 %) or 10 % FCS and antibiotics for human or rat cells, respectively. The experiments with TPA and CSC were performed in this medium one day later. In the experiments with lipoproteins the cells were exposed to lipoproteins in Ham's F10 medium containing 10 % lipoprotein deficient human serum (LPDS; Sigma) after a pretreatment with Ham's F10 and 10 % LPDS for 24 h, ensuring activation of the LDL receptor (Bierman and Albers, 1977).

Modulation of the LDL receptor activity was performed by a pretreatment of human smooth muscle cells with Ham's F10 medium containing 10% lipoprotein deficient human serum with or without 50 μg LDL-cholesterol/ml for 24 h. After pretreatment cells were incubated with LDL (0-200 μg LDL-cholesterol/ml) during 24 h followed by measurement of intercellular communication.

Intercellular communication was determined in subconfluent cell cultures after microinjection of a 20 % Lucifer Yellow CH (Sigma) (in 0.33 M Lithium Chloride) solution in a single cell close to the nucleus (Enomoto et al., 1984). In each smooth muscle cell culture at least 20 individual cells were microinjected using a vertical injection system (Olympus injectoscoop IMT-2-syf) (Yamamoto and Furusawa, 1978) with a dye filled capillary glass tip (Clark, Pangbourne, UK). The glass capillary tip was prepared by an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of 1 μm. The filled cells were checked with phase-contrast and fluorescense microscopy directly after microinjection. 15-20 min. after the first injection the number of communicating cells was determined. Photographs were made using an Olympus OM 2S camera using the injectoscope and a Kodak technical pan 2415 film.
Each experiment was performed in triplicate and at least two independent tests were done. The significance of the values was tested with a student t-test (Lindgren, 1976).

Cytotoxicity assay
2.10^5 cells were seeded into 24-well tissue culture plates (Costar). The next day the wells were rinsed with HBSS and the cells were (pre)incubated as described above. After the treatment 400 μl samples of the media were taken from each well and centrifuged. Lactate dehydrogenase (LDH) activity was measured in 100 μl supernatant. The maximum release of LDH was obtained by scraping the cells from the bottom after addition of 1 ml 0.5 % Triton X-100 in 0.1 M phosphate buffer (pH 7.5). After sonification for 5 min and centrifugation the LDH activity was measured in the supernatant (Mitchell et al., 1980). Each experiment was performed in triplicate.

RESULTS

Lucifer Yellow CH injected into single human or rat cells spread over an average over 22 and 6 surrounding cells, respectively (table 2.1). 24 h (Pre)treatment with medium containing 10 % lipoprotein deficient serum for 24 h resulted in the same intercellular communication as that produced by culture medium in both rat and human cells. The potent tumor promoting agent TPA (100 ng/ml) used as positive control caused an almost

Table 2.1 Number of fluorescent smooth muscle cells of human and rat after treatment with culture medium containing TPA, DMSO or (Ham's F10) medium with lipoprotein deficient serum (LPDS).

compound	time (h)	concentration	species	no. of fluorescent cells
-	-	-.-	human	21.9 ± 0.3
LPDS	24	10.0 %	human	20.5 ± 0.5
DMSO	4	0.1 %	human	19.2 ± 2.0
TPA	4	100.0 ng/ml	human	1.9 ± 0.1
-	-	-.-	rat	5.9 ± 0.6
LPDS	24	10.0 %	rat	5.5 ± 0.7
DMSO	4	0.1 %	rat	6.4 ± 1.3
TPA	4	100.0 ng/ml	rat	1.2 ± 0.2

Values are mean ± S.E.M.

complete inhibition after 4 h in smooth muscle cells of both species (table 2.1). The solvent DMSO (0,1 %) did not have an effect on the cell-cell communication.

The effects of lipoproteins and CSC on gap junctional communication were measured 24 h after the start of the incubation. No cytotoxic effects were seen at concentrations and treatment times of the compounds used in the experiments. Values of LDH activity after exposure to lipoproteins and CSC varied between 94% and 103% of the control values. When LDL was added to lipoprotein-deficient serum to achieve cholesterol concentrations of 50, 100 and 200 μg/ml of medium a dose-dependent decrease in communication in both human and rat smooth muscle cells was detected. The inhibition of cell-cell communication by LDL was the same for human and rat cells. The data are presented in Figs. 2.1 and 2.2. A significant inhibition of communication between arterial smooth muscle cells was present (30-35 %) at the lowest concentration of LDL tested in both species; the degree of inhibition was increased to about 45 % when the LDL-cholesterol concentration was raised to 100 μg/ml and increased to circa 55 % when the concentration of LDL-cholesterol was raised to 200 μg/ml. Fig. 2.2b shows the time course of the effect of LDL on intercellular communication. For human cells, significant inhibition of gap junctional communication occurred after 15 h and increased to 66 % after 48 h treatment with 100 μg LDL-cholesterol/ml ($p<0.005$). For rat cells, after 4 h

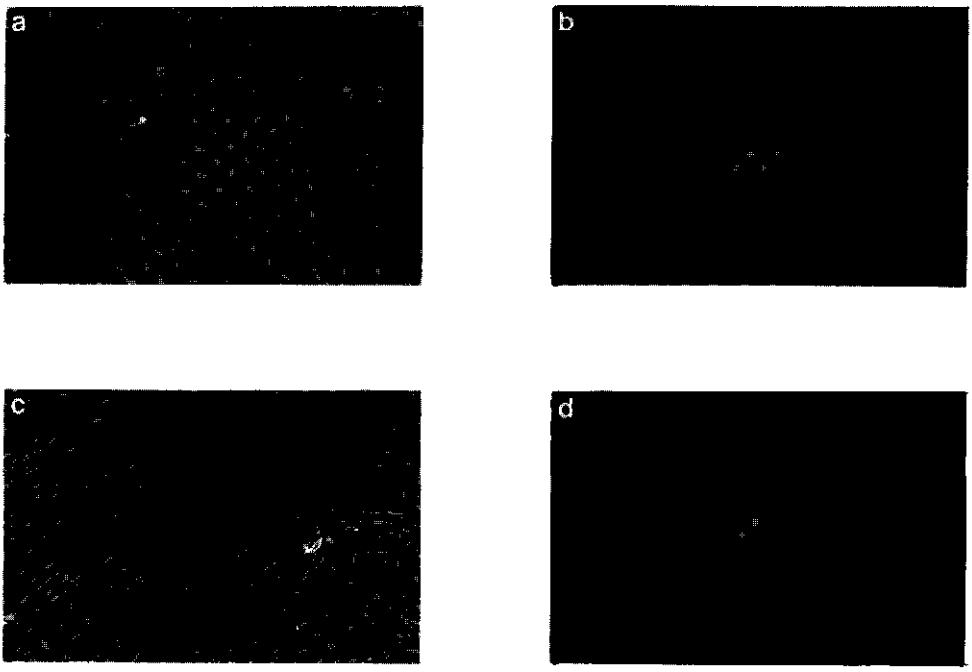

Fig. 2.1 Photomicrographs depicting Lucifer yellow transfer in cultures of human smooth muscle cells. Cells injected with Lucifer yellow dye are indicated by the arrows. Bar = 25 μm. (A) Phase contrast micrograph of untreated smooth muscle cells. (B) Lucifer yellow fluorescence in the same field. (C) Phase contrast micrograph of smooth muscle cells after exposure to 100 μg LDL-cholesterol/ml for 24 h. (D) Lucifer yellow fluorescence in the same field.

incubation of 100 μg LDL-cholesterol/ml, the intercellular communication was significant inhibited by 38 %. After 7.5 h the inhibition was maximal (45 %) and the inhibition persisted for another 40 h. So, the LDL-induced effects on human cells were enhanced after prelonged treatment in contrary to what happened in rat cells ($p < 0.025$ after 48 h). In contrast to LDL, HDL had no marked effect on the cell-cell communication. Smooth muscle cells of human and rat treated with HDL-cholesterol concentrations between 50 and 200 μg/ml for 24 h showed no significant differences in intercellular communication (Fig. 2.2b). Pretreatment of the human cells with 50 μg LDL-cholesterol/ml and subsequent addition of LDL levels between 0 - 200 μg cholesterol/ml resulted in an inhibition of intercellular communication, which was comparable with pretreatment with

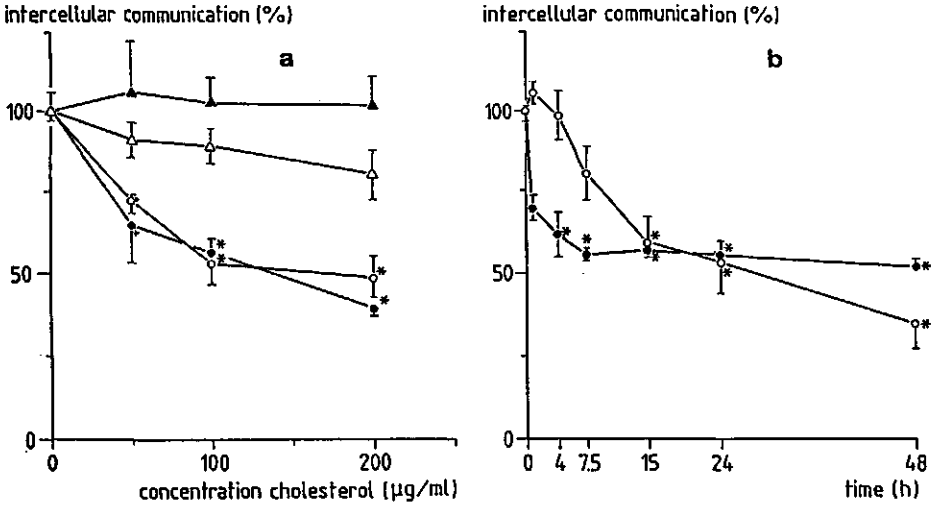

Fig. 2.2 Intercellular communication in smooth muscle cells of human and rat after exposure to different LDL or HDL concentrations for 24 h. (a) and for different periods at 100 µg cholesterol/ml (b). Values are mean ± S.E.M. Open symbols: human cells; closed symbols: rat cells; circles: LDL; triangles: HDL. * Significant different from controls, $p<0.005$, + Significant different from controls, $p<0.01$.

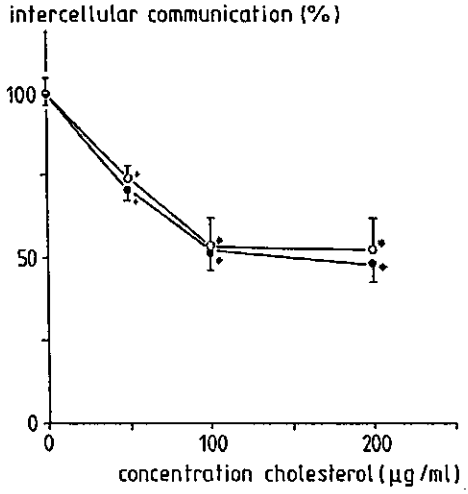

Fig. 2.3 Effect of LDL on intercellular communication in human smooth muscle cells with pretreatment (open circles) or without pretreatment (closed circles) with 50 µg LDL-cholesterol/ml for 24 h. Values are mean ± S.E.M. * Significant different from controls, $p<0.005$, + Significant different from controls, $p<0.01$.

lipoprotein deficient medium (Fig. 2.3).

Figure 2.4a shows that cigarette smoke condensate is also able to inhibit the communication between smooth muscle cells of human and rat. Exposure of smooth muscle cells to 27.5 μg CSC per ml for 24 h caused an inhibition of communication between human or rat cells of 11 % and 37 %, respectively. At 55 μg/ml the values increased to 28 % and 43 %, respectively; a further increase of the inhibition to about 50 % is observed when the concentration of CSC is raised to 110 μg/ml. At this concentration no differences were observed in CSC induced effects between human and rat smooth muscle cells. Fig. 2.4b shows the time course of the effects of CSC (55 μg/ml) on gap junctional communication in human and rat cells. After 4 h exposure to CSC the intercellular communication in human cells and rat cells is significantly inhibited by 32 % and 26 %, respectively. Exposure to CSC (55 μg/ml) for prolonged times (44 h) caused a further increase of inhibition by 65 % and 58 % in human and rat cells, respectively.

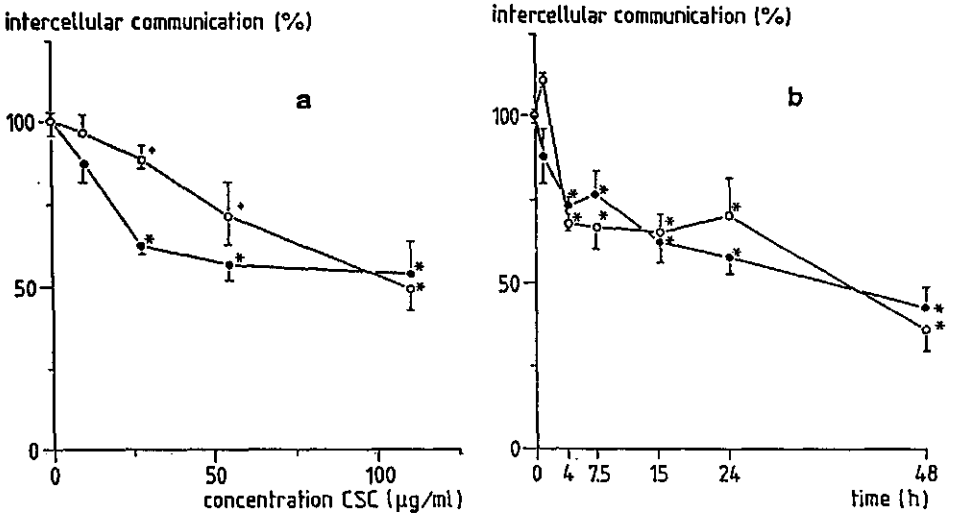

Fig. 2.4 Intercellular communication between smooth muscle cells of human and rat after exposure to different concentrations of Cigarette Smoke Condensate (CSC) for 24 h. (a) and for different periods at 55 μg/ml (b). Values are mean ± S.E.M. Open circles: human cells; closed circles: rat cells. * Significant different from controls, $p<0.005$, ⁺ Significant different from controls, $p<0.05$.

DISCUSSION

In this study intercellular communication was measured in smooth muscle cells of the human and the rat with the dye-transfer technique. In these cells cell-cell communication was almost completely blocked by the tumor promoter TPA, a known inhibitor of intercellular communication in various other cell types (Mosser and Bols, 1982; Enomoto and Yamasaki, 1985; Newbold and Amos, 1981; Rutten et al., 1988). The present study also revealed that CSC markedly reduced gapjunctional communication between smooth muscle cells as in fibroblasts and epithelial cells (Rutten et al., 1988; Hartman and Rosen, 1983). In addition LDL, in contrast to HDL, decreased intercellular communication in a dose-dependent way. Apparently not only the quantity of lipoproteins is of importance, but also the lipoprotein structure. The findings are consistent with epidemiological data linking high LDL (Smith and Slater, 1972) and low HDL (Glueck, 1976; Glueck et al., 1977; Gordon et al., 1977; Miller and Miller, 1975) plasma cholesterol concentrations to atherosclerosis.

We have investigated the hypothesis that the LDL-induced effects on intercellular communication are mainly receptor-mediated by modulating the LDL receptor activity. It was reported that activation of LDL receptors in smooth muscle cells occurs after preincubation with lipoprotein deficient medium (Bierman and Albers, 1977). Preincubation with LDL suppress the receptor binding activity (Bierman and Albers, 1977) and in case of a receptor-mediated process a subsequent addition of higher LDL levels should diminish the effects induced after pretreatment with lipoprotein deficient medium. We observed that the effect on intercellular communication was independent of LDL-pretreatment. This indicates that the inhibition on intercellular communication by LDL is mainly a non-receptor mediated process, possible by uptake of LDL via bulk phase endocytosis (Goldstein and Brown, 1974).

Intercellular communication is thought to be essential in the control of cellular homeostasis, growth and division (Loewenstein, 1979) and involves the passage of low molecular weight molecules and ions between adjacent cells via gap junctions (Simpson et al., 1977). Various substances which may be intimately related to the control of cell proliferation pass through the gap junction (Flagg-Newton and Loewenstein, 1979; Merk et al., 1972; Sheridan, 1974). It is proposed that the interchange of regulatory molecules

(chalones) across gap junctions can limit clonal expansion of (initiated) cells (Yotti et al., 1979a; Trosko et al., 1983). Blockage of the transfer of such regulatory factors between normal and initiated cells could lead to abnormal cell proliferation (Trosko et al., 1983; Yamasaki, 1987). The arterial smooth muscle initiated cells can be recruited to proliferate and form atherosclerotic lesions under conditions in which they are no longer affected by chalones. This may be one mechanism by which atherogens, enhance cell proliferation of intimal smooth muscle cells, ultimately leading to plaque formation. The intercellular junctions which are involved in carcinogenic mechanisms can also be involved in atherogenic mechanisms.

The development of an atherosclerotic lesion is characterised by a proliferation of arterial smooth muscle cells and an accumulation of free and esterified cholesterol, which is chiefly derived from plasma lipoproteins (Smith, 1974; Zilversmit, 1968), mainly LDL-cholesterol. Cholesterol is an important component of cellular membranes and incorporation of cholesterol and/or cholesterol (oxidation) derivatives in the membrane can cause dysfunction of the membrane (Sevanian and Peterson, 1986; Chang et al., 1988). Since gap junctions are membrane structures, changes in the membrane microenvironment could alter the functional capacity of gap junctions. This might be an explanation for the mechanism by which LDL induced inhibition of junctional communication. LDL is the major carrier of cholesterol towards the tissues, in contrast to HDL, which is a carrier of cholesterol from peripheral tissues to the liver. It cannot be excluded that other components of the lipoproteins than cholesterol(derivatives) contribute to the alterations in gap junctional communication. Further studies are needed to elucidate the mechanisms responsible for the LDL- and CSC-induced alterations of the gap junction mediated communication between smooth muscle cells.

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CHAPTER 3

MODULATION OF LOW DENSITY LIPOPROTEIN-INDUCED INHIBITION OF INTERCELLULAR COMMUNICATION BY ANTIOXIDANTS AND HIGH DENSITY LIPOPROTEINS

In order to study the capacity of antioxidants and high density lipoproteins (HDL) to modulate the effects of low density lipoproteins (LDL) on intercellular communication, arterial smooth muscle cells and a dye transfer method were used. LDL, in contrast to HDL, inhibited communication between human arterial smooth muscle cells of umbilical cords and human aorta in a dose-dependent way. LDL, which can be oxidized, as detected by lipid peroxidation assay and gel electrophoresis, did not influence cell-cell communication in the presence of antioxidants BHT, α-tocopherol and glutathione. These results suggest that LDL must undergo oxidative modification before it can influence cell-cell communication. Like antioxidants, HDL diminished LDL-induced inhibition of communication. This study suggests that modulation of gap junctional communication by the balance of HDL and LDL in plasma may influence atherogenesis.

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INTRODUCTION

Intercellular communication through gap junctions allows direct cell-to-cell transfer of compounds of low molecular weight, and may therefore play a role in the control of cell proliferation and differentiation and in tissue homeostasis (Gilula, 1977; Loewenstein, 1979; Yamasaki et al., 1984). The disruption of gap junctional communication by various chemicals is thought to be a factor in teratogenesis and the promotion of carcinogenesis and atherogenesis (Gilula, 1977; Warner et al., 1984; Weinstein et al., 1976; Zwijsen et al., 1990). This hypothesis is strongly supported by the fact that all tumor promoters that have been investigated block intercellular communication in cultured cells (Murray and Fitzgerald, 1979; Enomoto et al., 1981). In addition, it has been shown that transformed cells loose their ability to communicate with surrounding normal cells (Yamasaki et al., 1987). A number of oncogene products (e.g. those from *src* and *ras*), growth factors and viruses have also been shown to be associated with a decrease in gap junctional communication (Chang et al., 1985; Azarnia and Loewenstein, 1984; Madhukar et al., 1989).

It has been demonstrated that low density lipoproteins (LDL) also act as an inhibitor of intercellular communication (Zwijsen et al., 1990). Elevated plasma concentrations of these lipoproteins have been shown to be associated with accelerated atherogenesis (Tyroler, 1987; Goldstein and Brown, 1977; Steinberg, 1983). Recent studies have demonstrated that oxidative modification of LDL may play an important role in its atherogenic effect (Steinberg, 1988; Triau et al., 1988; Quinn et al., 1985; Steinberg et al., 1989). This oxidation occurs *in vivo* and *in vitro* and is totally inhibited by antioxidants, such as butylated hydroxytoluene (BHT) or vitamine E (Palinsky et al., 1989; Morel et al., 1984; Steinbrecher et al., 1984) resulting in blockage of several LDL-induced biological effects (Triau et al., 1988; Quinn et al., 1985; Steinberg et al., 1989). It has been shown that high density lipoproteins (HDL) can also prevent LDL-induced effects (Hessler et al., 1979). Accordingly, we have investigated the effect of antioxidants and HDL on the inhibition of gap junctional communication by LDL, using human arterial smooth muscle cells from different origins.

MATERIALS AND METHODS

Reagents

LDL, HDL and Lucifer Yellow CH were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Sodium pyruvate was obtained from BDH Chemicals Ltd (Poole, Dorset, UK) and NADH from Boehringer (Mannheim, Germany). BHT and DL-α-tocopherol were purchased from Merck (Darmstadt, Germany) and dissolved in 95% ethanol. Glutathione (GSH) was obtained from Janssen (Beerse, Belgium) and dissolved in Eagle's minimal essential medium (EMEM; Flow Labs, UK).

Cell culture

Smooth muscle cells were obtained from arteries of human umbilical cords and also from thoracic aorta of 56-58-yr-old men, for one microinjection experiment with lipoproteins. The adventitial layer was carefully removed by dissection. Intimal-medial explants (1 mm^2) were placed on coverslips, as described previously (Ross, 1971), and incubated in EMEM supplemented with 5% foetal calf serum (FCS; Gibco, U.K.), 5% human serum (Sigma), 50 μg gentamycin/ml and 1.5 μg amphotericin B/ml, in a 37 °C, 5% CO_2 humidified air environment. Sufficient peripheral growth to permit subcultures was obtained after 2-3 wk incubation, and all experiments were performed on cells in passage 2-3. These cells grow in a 'hills and valleys' pattern, and were characterized by raising fluoresceinated antibodies against smooth muscle actin (Chamley et al., 1977).

Experimental procedure

Cells were plated on glass coverslips, in 35 x 10-mm dishes, at a density of 2 x 10^5 cells per dish, and cultured in EMEM supplemented with FCS 5%, human serum (5%) and antibiotics. They were pretreated with Ham's F10 medium (Flow) containing 10% lipoprotein-deficient human serum (LPDS; Sigma) for 24 h, and exposed to lipoproteins and/or antioxidants.

Lipoproteins were isolated from fresh unfrozen human plasma (Sigma). Lipoprotein purity was controlled for breakdown and oxidation products as described previously (Zwijsen et al., 1990). Lipoproteins moved as a compact band and the absence of streaks as well as low level of malondialdehyde (MDA) in lipoprotein preparations indicated that lipo-

proteins were not oxidized essentially.

Measurement of cell-cell communication

This was performed in confluent cell cultures by the dye-transfer method (Enomoto et al., 1984). Briefly, fluorescent Lucifer Yellow CH (10% in 0.33 M-lithium chloride solution; Sigma) was injected through capillaries using a micro-injectoscope (Olympus), into a cell close to the nucleus. The capillaries were prepared by an automatic magnetic puller (tip diameter < 1 μm) (Narishige, Tokyo, Japan). During the injection, the cells were maintained at room temperature and the transfer to neighbouring cells was observed after 15-20 min. Since this dye cannot diffuse through the membrane, its transfer into neighbouring cells was believed to operate through gap junctional communication (Atkinson et al., 1985; Lo and Gilula, 1979; Stewart, 1978). In each cell culture, a minimum of 20 individual smooth muscle cells was injected; each experiment was performed in triplicate, and at least two independent experiments were done. Statistical analyses of the data were performed using Student's t-test.

Cytotoxicity assay

The cytotoxicity was determined by the measurement of lactate dehydrogenase released into the medium. Confluent cell cultures were exposed to lipoproteins, with or without antioxidants, or to antioxidants alone in 6-well dishes (Costar) for 24 h. 400 μl, out of a total volume of 2 ml, was taken from each well and centrifuged, and lactate dehydrogenase was measured in 100-μl supernatant (Mitchell et al., 1980). The maximal release of lactate dehydrogenase was obtained by scraping the cells with a rubber policeman in 1 ml Triton X-100 (0.5%) in 0.1 M phosphate buffer, and measuring the enzym activity in the supernatant after a 5-min sonification followed by centrifugation. Each experiment was performed in triplicate.

Lipid peroxidation assay

Lipid peroxidation was determined by the method of Yagi (1976). Confluent cell cultures were exposed to 100 μg LDL-cholesterol/ml, with or without antioxidants, in serum-free Ham's medium. After 24 h incubation, 100 μl of the incubate (without cells) was added to 1.5 ml thiobarbituric acid (0.67%) and 1.5 ml Cl_3CCOOH (20%). The mixture was

heated at 100 °C for 45 min, cooled with water, and immediately assayed fluorometrically using an Aminco-Bowman spectrophotofluorometer at excitation and emission wavelengths of 515 nm and 553 nm respectively. Freshly diluted malondialdehyde bis(dimethylacetal) (1,1,3,3-tetramethoxypropane; Aldrich, Steinheim, Germany) was used as a standard. The degree of LDL oxidation is expressed as nmol of MDA equivalents/mg LDL-cholesterol.

Electrophoresis

This was performed with 20 μl of the same incubates as previously. This volume was applied to each lane of the 1% agarose gel, which was then run in barbital buffer (Sigma) for 2 h, dried at 60 °C for 30 min, and lipid-stained with Sudan-black B. The gels were destained in a methanol-distilled water (2:1, v/v), rinsed in distilled water and dried. Electrophoretic mobility was evaluated by measuring the distance from the origin to the middle of the stained bands. The relative electrophoretic mobility was expressed as a ratio of the mobility of the sample to that of the control, which consisted of serum-free Ham's medium containing 100 μg LDL-cholesterol.

RESULTS

Figure 3.1 illustrates the effect of LDL and HDL on gap junctional communication between smooth muscle cells from thoracic aorta and umbilical cords. LDL decreased intercellular communication in both type of cells in a dose-dependent manner. However, umbilical cord cells respond to lower LDL-concentrations ($\geq$ 50 μg LDL-cholesterol/ml; $p \leq 0.05$) compared with aortic cells ($\geq$ 100 μg LDL-cholesterol/ml; $p \leq 0.01$). HDL did not influence gap junctional communication in either type of smooth muscle cells.

Table 3.1 shows the effects of the antioxidants BHT (20 μM), α-tocopherol (100 μM) and glutathione (1 mM) on the inhibition of intercellular communication by LDL. A 38% decrease in the communication was obtained by exposing arterial smooth muscle cells to LDL (100 μg LDL-cholesterol/ml) for 24 h. Simultaneous treatment of the cells with LDL and antioxidants did not inhibit cell-cell communication, whereas an inhibitory effect (-31%) was obtained by adding simultaneously oxidized LDL and BHT (20 μm) to the

Table 3.1 Effect of LDL with(out) antioxidants on intercellular communication.

incubation conditions	intercellular communication		
-	100.0	±	3.6
LDL	61.9*	±	6.5
LDL + BHT	90.8	±	10.6
LDL + α-tocopherol	98.9	±	9.7
LDL + glutathione	91.3	±	5.0
BHT	89.5	±	8.3
α-tocopherol	105.7	±	6.0
glutathione	105.1	±	4.9
oxidized LDL + BHT	68.8*	±	7.9

Smooth muscle cells derived from umbilical cords were incubated with LDL (100 μg LDL-cholesterol/ml) with or without BHT (20 μM), α-tocopherol (100 μM) or glutathione (1 mM) for 24 h. Oxidized LDL were obtained from LDL (100 μg LDL-cholesterol/ml) incubated with cells for 24 h. Intercellular communication was measured using a dye-transfer method; the number fluorescent cells in control cultures was approx. 21. Each number represents the mean of 120-180 injections from two independent experiments. Values are mean ± S.E.M. Asterisks indicate significant differences from controls (*$p \leq 0.01$).

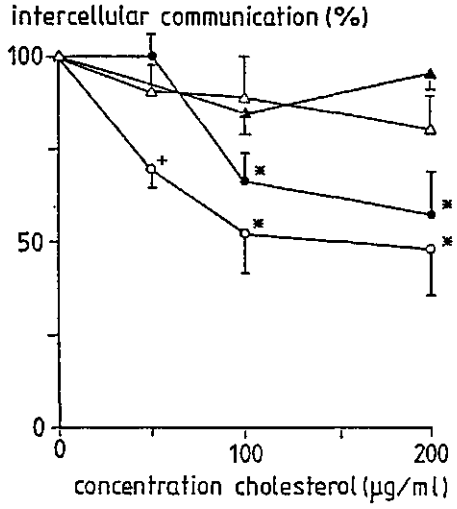

Fig. 3.1 Effect of Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL) on gap junctional communication between human smooth muscle cells derived from arteries of umbilical cords and thoracic aorta. Circles : LDL treatment; Triangles : HDL treatment. Open symbols : cells derived from umbilical cords. Closed symbols: cells derived from thoracic aorta. Each point represents the mean of 180-340 injections from three independent experiments. Values are mean ± S.E.M.; *$p \leq 0.05$, *$p \leq 0.01$

Table 3.2 Effect of antioxidants on the inhibition of intercellular communication by LDL.

Incubation conditions	TBARS (nmol MDA/ mg LDL-chol)	electro-phoretic mobility (mm)	relative electropho-retic mobility
LDL-control	0.0 ± 0.0	21 ± 1	1.00
SMC alone	1.2 ± 0.4	26 ± 2	1.25
BHT	0.1 ± 0.1	20 ± 1	0.95
α-tocopherol	0.3 ± 0.1	21 ± 1	1.00
glutathione	0.2 ± 0.1	20 ± 2	0.95
No-cell control	0.7 ± 0.3	24 ± 1	1.14

LDL (100 μg LDL-cholesterol/ml) was incubated in Ham's F10 medium with or without antioxidants (20 μm-BHT, 100 μm-α-tocopherol or 1 mM-glutathione), for 24 h. An aliquot of the medium was then assayed for the content of TBARS. A second aliquot was taken for the determination of electrophoretic mobility (i.e. the distance from the origin to the middle of the lipid-stained band). The relative electrophoretic mobility was expressed as the ratio of the mobility of the sample to that control LDL (no incubation). Values are means ± S.E.M. (n=3).

assay. BHT, α-tocopherol or glutathione, added alone to cell cultures, did not alter intercellular communication. The data showed that antioxidants might prevent completely the effect of LDL on cell-cell communication. At the LDL concentrations used, the lactate-dehydrogenase activity of the assay was 89-104% of the control value, indicating that the experiments were performed under non-cytotoxic conditions. The results from the lipid-peroxidation assay and gel electrophoresis are shown in Table 3.2. Incubation of smooth muscle cells with F10 medium containing LDL resulted in the appearance of thiobarbituric acid-reacting substances (TBARS) and an increase in the electrophoretic mobility of LDL. These biochemical changes were due to both the components of the medium and the smooth muscle cells. LDL electrophoretic mobility correlated well with the degree of oxidation of the lipoproteins as measured by the content of TBARS in the assay medium. The presence of antioxidants prevented the oxidation of LDL and therefore no inhibition of intercellular communication was observed. Figure 3.2 and Table 3.3 illustrate the influence of HDL on LDL inhibitory effect. Simultaneous treatment of the cells with HDL (20-80 μg cholesterol/ml) and LDL (100, 160 or 250 μg cholesterol/ml) significantly reduced ($p \leq 0.01$), but did not completely prevent the LDL-induced

Table 3.3 Restoration of HDL in LDL-induced inhibition of gap junctional communication (%).

HDL (μg cholesterol/ml)	LDL (μg cholesterol/ml)		
	100	160	250
0	0.0	0.0	0.0
10	15.4 ± 1.1	23.0 ± 2.6	7.2 ± 0.4
20	39.1 ± 3.3*	35.9 ± 6.6*	19.2 ± 0.9*
50	57.6 ± 7.3*	47.7 ± 5.0*	34.5 ± 1.4*
80	74.5 ± 8.9*	45.7 ± 2.1*	30.2 ± 0.4*

Intercellular communication was inhibited to 57.1±5.9, 45.3±5.2 and 37.4±3.3% by incubating smooth muscle cells with LDL at 100, 160 and 250 μg cholesterol/ml, respectively, for 24 h. The values in the table indicate the % of reduction in the inhibition of gap-junctional communication obtained by adding HDL simultaneously with LDL to the cultures before incubation. Each number represents the mean of 120-140 injections from two independent experiments. Values are means ± S.E.M. Asterisks indicate significant differences from controls (*p≤0.01).

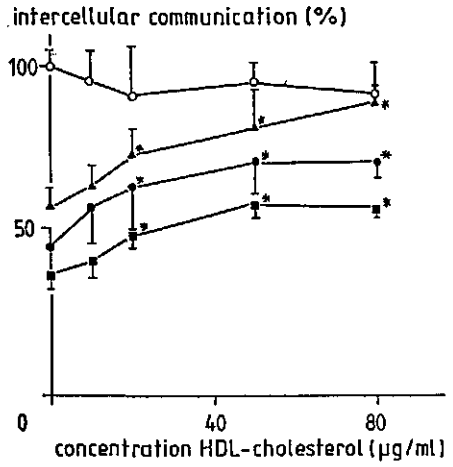

Fig. 3.2 Effect of combinations of High Density Lipoproteins (HDL) and Low Density Lipoproteins (LDL) on inhibition of gap junctional communication between human smooth muscle cells. Open symbol: no LDL treatment. Closed symbols : LDL treatment ; triangles : treatment with 100 μg LDL-cholesterol/ml; circles : treatment with 160 μg LDL-cholesterol/ml; squares : treatment with 250 μg LDL-cholesterol/ml. Each point represents the mean of 120-140 injections from two independent experiments. Values are mean ± SEM. * p≤0.01 compared with LDL treatment alone.

inhibition of intercellular communication. The effect of HDL increased in a dose-dependent manner between 0 and 50 μg HDL-cholesterol/ml; it reached saturation levels at 80 μg HDL-cholesterol/ml when combined with 160-250 μg LDL-cholesterol/ml. Exposure of the cells to the highest HDL dose (80 μg cholesterol/ml) and increasing LDL doses (100, 160 and 250 μg cholesterol/ml) led to a decrease in the LDL-induced inhibition of intercellular communication by 75, 46 and 30% respectively.

DISCUSSION

Smooth muscle cells from arteries of human umbilical cords and the thoracic aorta responded in the same manner to LDL and HDL as far as intercellular communication is concerned. As previously reported (Zwijsen et al., 1990), the present study shows that LDL, in contrast to HDL, inhibited intercellular communication in a dosis-dependent manner. The simultaneous addition of HDL and LDL to the assay reduced the inhibitory effect of LDL on cell-cell communication. This result suggests that the balance between LDL and HDL plays an important part in the final effect of intercellular communication. This observation is consistent with a number of epidemiological studies indicating that high LDL and low HDL concentrations in plasma are associated with the development of accelerated atherosclerotic vascular disease (Glueck et al., 1977; Gordon et al., 1977; Miller and Miller, 1975; Smith and Slater, 1972).

The data also show that oxidation of LDL plays an important role in influencing gap junctional communication. This reaction is due both to compounds of F10 medium that can catalyse peroxidation (i.e. 0.01 μM-Cu and 3.0 μM-Fe) and to smooth muscle cells. In assays using lipoprotein-deficient serum, the levels of oxidized LDL were much lower than those reported in previous studies (Morel et al., 1984; Steinbrecher et al., 1984). LDL oxidation was prevented by antioxidants, such as BHT, DL-α-tocopherol and glutathione. This result is in agreement with other studies (Morel et al., 1984; Palinsky et al., 1989). In addition, it has been reported that HDL can also inhibit the oxidative modification of LDL (Parthasarathy et al., 1990). Our study shows that there is a clear relationship between LDL oxidation and inhibition of intercellular communication: LDL that had been oxidized prior to incubation inhibited intercellular communication; a

combination of HDL and LDL reduced LDL-induced effects on cell-cell communication; and all the three antioxidants used (BHT, α-tocopherol and glutathione) completely prevented the LDL inhibitory effect. These findings suggest that LDL must undergo oxidative modification before it can influence cell-cell communication. A previous study has also shown that LDL at 100 μg cholesterol/ml inhibited cell-cell communication after at least 7.5 h incubation (Zwijsen et al., 1990). It has been suggested that this retarded effect is possibly caused by intrinsic antioxidants as α-tocopherol. LDL, therefore, must loose its antioxidants before it undergoes oxidative modification and subsequently acquires its inhibitory property (Esterbauer et al., 1987).

In vivo data have also demonstrated that LDL oxidative modification plays an important role in atherosclerosis (Haberland et al., 1988; Palinsky et al., 1989). Using immunocytochemical techniques, the authors have shown that oxidation of LDL occurs *in vivo* and there is an accumulation of oxidized LDL in atherosclerotic lesions. These products of lipid peroxidation appear in the form of ceroids and, compared with normal areas, are present in greater concentrations in atheromatous plaques (Mitchinson et al., 1985). Furthermore, the role of oxidized LDL in atherosclerosis is further supported by experiments showing that antioxidant therapy slows the progress of early atherosclerotic lesions in receptor-deficient rabbits (Kita et al., 1987).

The nature of LDL reactive metabolites has not yet been determined. Our results show that the simultaneous addition of BHT and oxidized LDL did not modify the inhibition of intercellular communication. This finding suggests that the inhibitory effect observed is due to oxidized derivatives of LDL and not to free radicals or intermediate substances generated during the peroxidative process. The inhibitory compounds may result from the oxidation of cholesterol and/or degradation of polyunsaturated fatty acids (Jurgens et al., 1986). It has been shown that the oxidation of these fatty acids leads to the formation of shorter molecules such as malonaldehyde and 4-hydroxynonenal. These aldehyde can bind covalently to apolipoprotein B principally through lysine residues, and therefore modify the structural and biological properties of LDL (Esterbauer et al., 1987; Steinberg, 1988). These alterations lead to an incapacity of the apolipoprotein to recognize LDL receptors in smooth muscle cells. This is in agreement with previous findings showing that the LDL inhibitory effect is mainly non-receptor mediated (Zwijsen et al., 1990).

It has been shown that compounds that are associated with atherosclerosis (e.g. high levels

of oxidized LDL, low levels of HDL and cigarette-smoke condensate) inhibit the communication between vascular smooth muscle cells (Zwijsen et al., 1990). The disruption of the growth control by these compounds results in an uninhibited multiplication of these cells leading to the formation of atherosclerotic lesions. Modulation of intercellular communication therefore may be an essential step in the pathogenesis of atherogenesis. We have also demonstrated that umbilical cord cells can be used to investigate gap junctional communication between human arterial cells.

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CHAPTER 4

EFFECT OF CHOLESTEROL AND OXYSTEROLS ON GAP JUNCTIONAL COMMUNICATION BETWEEN HUMAN SMOOTH MUSCLE CELLS

Intercellular communication is considered to play an essential role in maintaining and controlling cell growth, cell differentiation and homeostasis. Cell-cell communication can be regulated by factors that influence gap junctional function. In this study it was demonstrated that cholesterol and oxidized cholesterol have the potential to modulate gap junctional communication between human smooth muscle cells in an opposite way. Cholesterol supplementation to human smooth muscle cells resulted in an increase of gap junctional communication up to 130% with regard to the control values. However, autooxidized cholesterol inhibited gap junctional communication more than 40%. Testing of several pure cholesterol oxidation derivates on gap junctional communication demonstrated that all of them were capable to inhibit intercellular communication in the order 25-hydroxycholesterol > cholestan-3ß,5α,6ß-triol > 7-ketocholesterol > cholesterol 5,6 α-epoxide. The cell-cell communication inhibiting potency of these oxysterols are in accordance with their atherogenic potency. This implies that cholesterol oxidation products, in stead of pure cholesterol, can be promoting factors in the atherogenesis by influencing gap junctional communication between arterial smooth muscle cells, the target cells of atherosclerotic lesions.

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INTRODUCTION

Intercellular communication mediated via gap junctions is essential to allow coordination and control of the proliferation, differentiation and activities of cell populations (Loewenstein, 1979). Gap junctions have been shown to be involved in a multitude of cellular processes ranging from cell synchronization and neuronal function to cell differentiation and carcinogenesis (Loewenstein, 1979; Spray and Bennett, 1985; Pitts and Finbow, 1986). Moreover, from previous studies the conclusion could be drawn that these processes may also play an essential role in the development of atherosclerosis. It was demonstrated that cigarette smoke condensate and carriers of cholesterol and lipids in the blood (low density lipoproteins (LDL) and high density lipoproteins (HDL)) markedly influence cell-cell communication, which correlates with their association for atherosclerosis (Zwijsen et al., 1990; Zwijsen et al., 1991).

Generally in studies on experimental atherosclerosis cholesterol is used to induce atherosclerotic lesions. However, there is also evidence that probably not cholesterol, but its oxidation products have an atherogenic potential (Van Lier and Smith, 1967/1970; Smith et al., 1967; Addis and Park, 1989). In rabbits alimentary hypercholesterolemia led to increased levels of lipid oxidation products and to depression of antioxidants systems of blood (Ledwozyw et al., 1986; Voskresensky, 1981). A diet poor in antioxidants or containing lipid oxidation products accelerated the development of experimental atherosclerosis (Voskresensky, 1981). There is also a great deal of evidence showing that oxidative modification of LDL, the main carrier of cholesterol, is an essential process in atherogenesis. LDL-oxidation is accompanied by the appearance of lipid oxidation products (Steinbrecher et al., 1984; Goldstein et al., 1979). Additional evidence for the role of oxidative processes in atherosclerosis are the accumulation of lipid peroxides in blood and arterial wall of patients with atherosclerotic alterations in blood vessels (Gray et al., 1971; Lopukhin et al., 1983; Ledwozyw et al., 1986). In the mean time studies have been done with individual cholesterol oxidation products, which show that these compounds are far more atherogenic

than cholesterol itself (Tayler et al., 1979; Shih, 1980). Consequently, the conclusion can be drawn that the deposition of cholesterol and its esters in atherosclerotic lesions is probably not direct responsible for initiation of the lesion, but is merely a secondary process (Peng et al., 1985 a/b).

Cholesterol oxidation products are formed both in food as well as endogenously in the body tissues. They have been identified in certain processed food, like dried egg products, powdered milk, cheese and in a variety of high temperature dried animal products (Chicoye et al., 1968; Naber and Biggert, 1982; Bascoul et al., 1986; Addis, 1986). On the other hand, the in vivo formation of oxysterols occurs via microsomal and mitochondrial cytochrome P-450 dependent lipidperoxidation and lipid autooxidation (Aringer and Eneroth, 1974; Martin and Nicholas, 1973; Sevanian et al., 1985). So, in view of the common occurence of oxysterols, they may well represent a risk factor in atherosclerotic cardiovascular disease. Sofar, the initiating effects of these compounds received more attention than their promoting effects in atherogenesis. The main object of the present study was therefore to examine cholesterol, autooxidized cholesterol and major known oxysterols including cholesterol 5,6 α-epoxide, 25-hydroxycholesterol, cholestan-3ß,5α,6ß-triol and 7-ketocholesterol with respect to their effects on intercellular communication between human smooth muscle cells, a probable promoting mechanism.

MATERIALS AND METHODS

Materials

Cholesterol, oxysterols, essentially fatty acid-free bovine serum albumin and Lucifer Yellow were purchased from Sigma Chemicals Co (U.S.A.). The purity of cholesterol and oxysterols was confirmed by thin-layer chromatography using ethylacetate-heptane (1:1; by vol.) with single spots and these compounds were stored under nitrogen.

Cell culture

Human smooth muscle cells obtained from arteries of umbilical cords (Zwijsen et al., 1990) were cultured in Eagle's Minimal Essential Medium (EMEM; Flow Laboratories, U.K.) supplemented with 10% fetal calf serum (Gibco, U.K.), gentamycin (50 μg/ml; Gibco) and amphotericin B (1.5 μg/ml; Gibco) in a 37 °C, 5% CO_2 humidified air environment.

Cholesteroloxidation and supplementation

Cholesterol was oxidized by air at 65 °C during 5 weeks. Oxidation of cholesterol was confirmed by thin-layer chromatography. The compounds were dissolved in 96% ethanol and added to EMEM containing 10% lipoprotein deficient serum (LPDS; Sigma) and antibiotics. During 24 h cell cultures were exposed to this medium. In case of (oxidized) cholesterol treatment 5% fatty-acid free bovine serum albumin (BSA) was included in the culture medium 24 h prior and during exposure. Control cultures received vehicle and solvent without sterols.

Cytotoxicity assay

Cytotoxicity tests were carried out in order to avoid interference of cytotoxicity and inhibition of intercellular communication. Therefore, smooth muscle cells ($2x10^5$) were seeded into 24-well tissue culture plates and (pre)treated with oxysterols (1-10 μM) or cholesterol(mix) (50-400 μg/ml) as cited above the next day. After exposure cytotoxicity was determined morphologically and by measuring of lactate dehydrogenase (LDH) in cells and medium as described previously (Mitchell et al., 1980). Each experiment was performed in three wells and at least two independent tests were done. It was found that values of LDH activities after treatment varied between 94% and 102% of the control values. No morphologically changes were seen at concentrations of the compounds used here. So, we tested these compounds under non-cytotoxic conditions.

Intercellular communication studies

Intercellular communication experiments were performed as following. Glass capillaries with a tip diameter of 1 μm prepared with a dual-step puller (Narishige Co., Tokyo, Japan) were filled with a 20% Lucifer Yellow CH solution dissolved in 0.33 M lithium chloride and fixed at an inverted microscope (Olympus Injectoscope IMT-2 SYF). The cells were microinjected close to the nucleus. The extent of intercellular communication was determined by the number of fluorescent neighboring cells scored under a fluorescence phase-contrast microscope 15 min after injection. In each dish containing confluent smooth muscle cell culture at least 20 individual cells were injected. Each experiment was performed in three dishes, while at least two independent tests were done.

Statistical treatments

Values were routinely expressed as means with standard deviations (SD). To determine the statistical significance of values obtained with the various treatments, Student's t-test was applied.

RESULTS

In a first series of experiments pure cholesterol was compared with cholesterol in oxidized form. As can be seen in Fig. 4.1 cholesterol even stimulates dye transfer between human smooth muscle cells with a maximum of 130% communication at 200 μg cholesterol/ml ($p \leq 0.005$); At 400 μg cholesterol/ml the enhanced communication was persisted ($p \leq 0.05$). However, cholesterol which was oxidized by air at 65 °C for 5 weeks reacted oppositive to cell-cell communication. The mix of cholesterol derivates caused inhibition of communication between the smooth muscle cells in a dosis-dependent manner. Exposure of 100 μg cholesterol derivates mix/ml during 24 h showed an inhibition of 24 % and at the highest cholesterol mix concentration used here (400 μg/ml) the inhibition was increased to 42 %

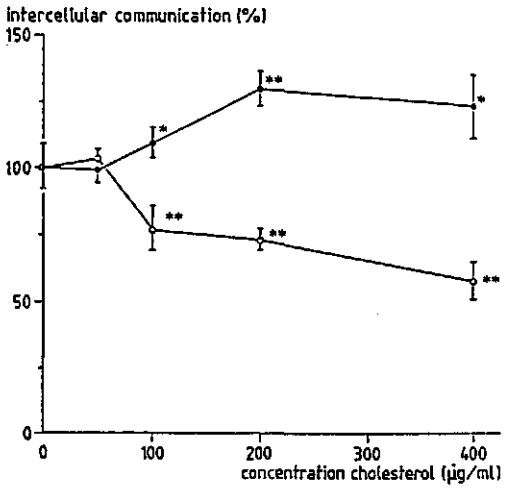

Figure 4.1 Effect of cholesterol and autooxidized cholesterol on gap junctional ommunication between human smooth muscle cells. •: cholesterol treatment; o: autooxidized cholesterol treatment. Each point represents the mean of 120-240 injections from two independent experiments. Values are mean ± S.D.; * $p \leq 0.05$, ** $p \leq 0.005$.

($p \leq 0.005$). In a second series of experiments the identified oxysterols including cholestan-3ß,5α,6ß-triol, 25-hydroxycholesterol, cholesterol 5,6 α-epoxide and 7-ketocholesterol were tested on their effects on gap junctional communication. As shown in Fig. 4.2 all of them were able to reduce intercellular communication. Cholestan-3ß,5α,6ß-triol was tested between 0.1 tot 10 µM and caused an inhibition of cell-cell communication at concentrations above 1 µM ($p \leq 0.005$). Cholestan-3ß,5α,6ß-triol at 1 µM and 10 µM influence the gap junctional communication to 72% and 51% communication with regard to the control values, respectively ($p \leq 0.005$). Another oxysterol, 25-hydroxycholesterol, also influences intercellular communication in a dosis-dependent manner. After treatment with 10 µM 25-hydroxycholesterol there was a communication of 17% left ($p \leq 0.005$).

The effects of 7-ketocholesterol on communication between smooth muscle cells at 1 µM, 5 µM and 10 µM was 96%, 80% and 56% with regard to the control values, respectively.

In this concentration range cholesterol 5,6 α-epoxide did influence the intercellular communication to a lesser extent. The maximal influence on communication induced by cholesterol 5,6 α-epoxide was a reduction of 20% ($p \leq 0.05$); this extent of reduction occurred at 10 μM cholesterol 5,6 α-epoxide. Resuming the influence of several oxysterols on gap junctional communication, it can be concluded that all of the compounds used in this study were able to reduce communication in the order 25-hydroxycholesterol > cholestan-3,5,6-triol > 7-ketocholesterol > cholesterol 5,6 α-epoxide.

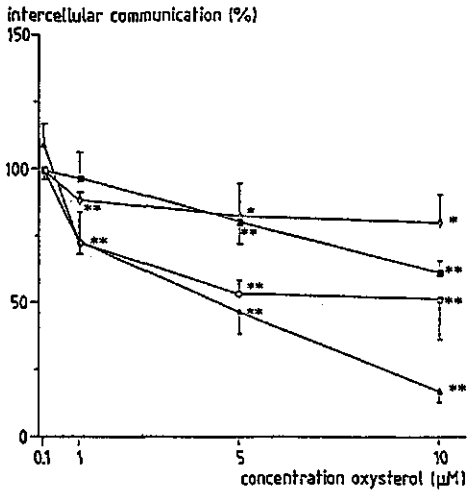

Figure 4.2 Effect of several oxysterols on gap junctional communication between human smooth muscle cells. ♦: cholesterol 5,6 α-epoxide; o: cholestan, 3ß,5α,6ß-triol; ▲: 25-hydroxycholesterol; ■: 7-ketocholesterol. Each point represents the mean of 120-180 injections from two independent experiments. Values are mean ± S.D.; * $p \leq 0.05$; ** $p \leq 0.005$.

DISCUSSION

The data presented in this report clearly show that exogenous cholesterol can stimulate gap junctional communication between human smooth muscle cells. This effect was demonstrated

previously in hepatoma cells (Meijer et al., 1990; Malewicz et al., 1990), but it could not be shown in V79 fibroblasts (Chang et al., 1988), possibly because of differences in cell types and/or cholesterol concentrations used in the last study. It is well known that cholesterol is an important structural and functional substituent of the cell, especially by moderating the fluidity of the membrane (Papahadjopoulos, 1974). Cholesterol prevents the crystallization of fatty acyl chains by fitting between them. An opposite effect of cholesterol is to sterically block large motions of fatty acyl chains and thereby make the membrane less fluidity (Papahadlopoulos, 1974). In addition, gap junctions appear to be characterized by a high level of cholesterol (per mg protein) and by a high cholesterol-to-phospholipid molar ratio (Meijer et al., 1990). So, an explanation for the observed cholesterol-induced stimulation of gap junctional communication could be that cholesterol is required as an essential structural component for the function of gap junctions.

In contrast to cholesterol, a mix of autooxidized cholesterol appeared to inhibit the gap junctional communication. Testing of several pure oxysterols increased the evidence that cholesterol in oxidized form is capable to inhibit intercellular communication. It was demonstrated that all of the oxysterols were capable to reduce intercellular communication in the order 25-hydroxycholesterol > cholestan-3ß,5α,6ß-triol > 7-ketocholesterol > cholesterol 5,6 α-epoxide. The order found with regard to inhibition of intercellular communication equals the reported atherogenic potential (Peng et al., 1985b). Thus, the outcome of this study fitt very well in the hypothesis that intercellular communication is involved in atherogenesis. Disturbance of intercellular communication by these compounds may result in the disturbance of controlling cell growth, differentiation and homeostasis (Loewenstein, 1979). Since this control system is absent in atherosclerotic plaque cells this can be one mechanism in the promotion of atherogenesis. Therefore, it is relevant to consider in further detail the role of oxysterols in membrane and gap junctional functions.

The incorporation of exogenous oxysterols into plasma membranes of cultured cells has been demonstrated repeatedly. In the membrane insertion hypothesis it is envisaged that exogenous oxysterol be incorporated into membranes, displacing membrane cholesterol, with

accompanying effects on membrane stability and function. Oxysterols possess by a combination of inhibition of cholesterol uptake from exogenous sources and inhibition of cellular biosynthesis of cholesterol also the capacity to render cells "cholesterol-starved". Inhibitory effects of oxysterols on 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co A) reductase activity and cholesterol biosynthesis were reported in cultured smooth muscle cells, with 25-hydroxycholesterol as the strongest inhibitor (Peng et al., 1979). Other important effects of cholesterol oxidation derivates are stimulation of a soluble acid cholesterol ester hydrolase, which catalyzes the hydrolysis of cholesteryl esters (Smith et al., 1974) and inhibition of the uptake of cholesterol (Bing and Sarma, 1975; Peng et al., 1985a). Depletion of cellular cholesterol by these processes and oxysterol insertion into membranes diminishes fluidity of fatty acid chains of phospholipids within the membranes (Wharton and Green, 1982; Rooney et al., 1986; Verma et al., 1983).

Structural studies show that a gap junction consists of two opposed cylindrical units, called connexons, each made up of six protein subunits, which delineate an axial aqueous pore (Caspar et al., 1977; Unwin and Zampighi, 1980). The effective diameter of gap junction channels are performed by a coordinated sliding and tilting motion of the protein subunits, which are typically aligned in a helix-like configuration. The fluidity of the acyl chain probably provides the required freedom of motion, allowing proteins within the membranes to undergo conformational changes, rotational and/or translocational movements associated with their activity. Oxysterols diminish fluidity of acyl chains (Verma et al., 1983; Rooney et al., 1986) and thereby possibly influence the rotational changes of the subunits of the gap junctions resulting in the observed reduction of the intercellular communication.

The effects of oxysterols acting on cell membranes may involve yet another fundamental process for several oxysterols, that of Ca^{2+} metabolism. The permeability of gap junctions is regulated by intracellular levels of the calcium ion. Elevation of the intracellular level of Ca^{2+} leads to a (graded) closure of gap junctions. Oxysterols can modulate Ca^{2+} influx and efflux in cultured cells in different ways, e.g. cholestan-3ß,5α,6ß-triol, 7-ketocholesterol or 25-hydroxy-cholesterol exposure caused an increased Ca^{2+} influx (Holmes et al., 1986;

Sevanian and Peterson, 1986). However, Ca^{2+} efflux is increased by cholesterol 5,6 α-epoxides (Sevanian and Peterson, 1986). So, oxysterols are not always active in the same way and can modulate gap junctional communication in different manners. Besides affecting Ca^{2+} channels oxysterols may influence Ca^{2+} metabolism also via inhibition of the protein calmodulin, which is implicated in a variety of Ca^{2+}-dependent events (Tipton et al., 1987 a,b). This inhibition would have the effect of suppressing actions of Ca^{2+} on intracellular processes, including the Ca^{2+} calmodulin kinase inhibiting HMGCoA reductase and thereby de novo sterol biosynthesis (Beg et al., 1987 a,b) resulting in decreased membrane fluidity and gap junction dysfunction.

It is of great interest and importance that these spontaneously occuring cholesterol oxidation products occur in many cholesterol-containing food items. Some of them were found to be easily absorbed and selectively transported in very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in the blood and ultimately they can be taken up by the target cells of atherosclerosis, the smooth muscle cells (Peng et al., 1982). In addition, the present study demonstrated that oxysterols markedly influence gap junctional communication at physiological conditions. Therefore, oxysterols may be important promoting factors in the atherogenesis.

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CHAPTER 5

THE PLATELET-DERIVED GROWTH FACTOR CHAIN A GENE IS ONE OF THE TRANSFORMING GENES PRESENT IN HUMAN ATHEROSCLEROTIC LESIONS

DNA derived from human aortic plaques has transforming potential. Mice C3H/10T1/2 cells transfected with human plaque DNA resulted in 0.070 foci/μg DNA. The transforming elements in plaque DNA can be transmitted serially and the transformed cells are tumorigenic *in vivo*. The presence of *Alu* positive signals in primary and secondary transformants indicates the presence of human DNA. In this report we demonstrate that platelet-derived growth factor chain A (PDGF-A) activation can contribute to the pathogenesis of the atherosclerotic plaque. However, the PDGF-A gene is responsible for only one of the seven plaque DNA-induced cell transformations screened in the present study, which emphasizes the multifactorial nature of atherosclerosis.

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Renate M.L. Zwijsen, Anne Mensink, Leonie H.E. Kockx, Rommert C. van den Bos and Jan H. Koeman. Platelet-derived growth factor chain A is one of the transforming genes present in human atherosclerotic lesions (in preparation).

INTRODUCTION

Atherosclerotic lesions occur in the innermost layer of the artery wall and consist mainly of proliferated smooth muscle cells surrounded by large amounts of connective tissue, numerous lipid-loaden macrophages and varying numbers of lymphocytes. Benditt and Benditt (1973) showed that human atherosclerotic plaques are monoclonal in origin, providing an explanation for the proliferation of smooth muscle cells. In their view cells of a plaque are the progeny of a single mutated smooth muscle cell near the site of a plaque, which is transformed by mutational or viral events. So, they compared atherosclerotic lesions with benign smooth muscle cell tumors of the arterial wall. In favor of this hypothesis it was shown that several chemical mutagens (e.g. polycyclic aromatic hydrocarbons) can induce atherosclerotic lesions in laboratory animals (Revis et al., 1984; Bond et al., 1981; Albert et al., 1975). Furthermore, Majesky et al. (1985) showed that focal proliferation of intimal smooth muscle cells can be induced by an initiation-promotion treatment sequence, analogous to the multistage mechanism demonstrated for tumor formation in a wide variety of animal tissues. Treatment with other kinds of cancer causing agents like oncogenic viruses (Fabricant et al., 1978) and radiation (Gold, 1961) also induced atherosclerosis.

The hypothesis of atherosclerotic lesions being similar with benign smooth muscle cell tumors of the arterial wall implicates an activation of one or more dominantly active oncogenes or the loss or inactivation of an active anti-oncogene in plaque cells. The involvement of transforming genes in atherogenesis was demonstrated by Penn et al. (1986) and Ahmed et al. (1990). These authors demonstrated that transfection of human atherosclerotic plaque DNA resulted in a transformation of NIH 3T3 cells. However, Yew et al. (1989) failed to observe transforming activity of human plaque DNA in their NIH 3T3 cell transformation assay experiments, suggesting that the activation of a transforming gene is not a general feature of plaque formation. Regarding the results of the experiments pertaining to the monoclonal origin of atherosclerotic lesions we decided to search for active transforming gene(s) in human atherosclerotic plaques using atherosclerotic lesions of a different origin (human lesions of the aorta in stead of coronary artery) and another cell type (C3H/10T1/2) and possibly to identify them. Here we show that also human aortic atherosclerotic plaque DNA also contains active trans-

forming genes. This study also indicates that the platelet-derived growth factor chain A (PDGF-A) gene may play an important role in the atherogenesis as one of the several transforming genes present in plaque DNA.

MATERIALS AND METHODS

DNA samples and transfection

Human DNA samples were obtained from lesions of the thoracic aorta and from normal thoracic aorta by autopsy of men aged 50-69, who had no cancer and no diabetes mellitus history. Portions of tissues from five persons were pooled (plaque A, B, C) or several plaques derived from one person were pooled (a,b,c,d). DNA from normal aorta, umbilical cord artery and C3H/10T1/2 cells were used as negative controls and C3H/10T1/2 cells transformed by 3-methylcholantrene (MCA) served as a positive control. The tissues were minced and pottered in ice-cold 0.5% NaCl, 5mM EDTA (pH 7.5) and 3% 4-aminosalicylic acid (BDH, Poole, UK) until complete disruption of tissue; cell monolayers were scraped with a rubber policeman, pelleted and resuspended in a small volume of 10 mM Tris/1mM EDTA (pH 7.5).

DNA isolation procedures were carried out by phenol extractions, proteinase K (Merck, Darmstadt, Germany) and RNase A (Sigma, St Louis, MO) treatments. Precipitation of DNA was performed by 0.4 volumes of 5 M ammoniumacetate followed by 2 volumes of isopropanol at room temperature. Precipitated DNA was dissolved in 10 mM Tris/0.2 mM EDTA (pH 7.5). High molecular weight DNA was used for DNA-mediated transfer assays with C3H/10T1/2 clone 8 cells (passage 9-12). C3H/10T1/2 clone 8 cells are mice fibroblasts (American Type Culture Collection) that were propagated in Eagle's Minimal Essential Medium (Flow Laboratories, U.K.) with 10% fetal calf serum (FCS; Gibco, UK). Exponentially growing cells were transfected using the electroporation technique. Therefore, 1 x 10^7 C3H/10T1/2 cells/ml in phosphate buffered saline (PBS) and 20 or 40 μg DNA in a cuvette with aluminium electrodes were placed on ice for 10 minutes allowing the DNA stick to the cells. Electroporation was carried out at 2000 V for 0.9 seconds. After another 10 minutes on ice the cells were resuspended in Eagle's Minimal Essential Medium (EMEM; Flow laboratories, UK) and 10 % FCS. The cells were

cultured for four to five weeks in EMEM, 10 % FCS and gentamycin (50 μg/ml) and were scored for type III foci (Reznikoff et al., 1973). Foci were picked up by regional trypsination or were fixed with methanol and stained with 5% Giemsa. The isolated foci cells were grown in mass culture followed by DNA isolation for a second round of transfection.

Southern blot analyses

DNAs were digested with the restriction enzymes Eco RI or BamH I (Life Technologies Inc., Gaithersburg, USA), separated by electrophoresis in horizontal agarose gels and transferred to Gene Screen + membrane filters (NEN, Boston, USA). The blots were hybridized using the following cloned DNA fragments as probes: human *c-sis*, pSM1 (Josephs, 1984) and human PDGF A chain, clone D1 (Bertholtz et al., 1986); probes of *v-mos*, *c-myc*, *v-Ha-ras*, *N-ras*, *v-Ki-ras*, *fos* and the human repetitive *Alu* sequence (a 300 bp BamHI insert of pBLUR8) were purchased from ONCOR (Gaitherburg, MD). The fragments were ^{32}P-labeled by random priming (Feinberg and Vogelstein, 1983). After hybridization the filters were washed at 65 °C in 0.5 x SSC or 0.1 x SSC (1 x SSC = 0.15 M sodium chloride / 0.015 M sodium citrate, pH 7) and 1% SDS (BDH, Poole, UK). Autoradiography was performed with X-ray film (Kodak X-Omat) and Lightning Plus Intensifying screens (Dupont) for 2-7 days at -80 °C.

Tumorigenicity

Tumorigenicity of transformed cell lines was determined in male Sprague Dawley athymic nu^+/nu^+ mice. Cultured transformed cells were trypsinated and resuspended in EMEM. The volume was adjusted to a density of 10^7/ml; 0.2 ml of cell suspension was injected subcutaneously in the scapular area using a 23 gauge needle. All animals were bred and housed under specific pathogen free conditions and were monitored during 10 weeks for tumor development. After sacrificing, samples of the tumors were examined histologically.

RESULTS

The transforming potential of DNA derived from human atherosclerotic lesions was tested by DNA-mediated gene transfer. Transformed cell clones (foci) arose in cells transfected with each sample of (atherosclerotic) tissue DNA pooled from five persons (plaque A,B,C) and with each sample of pooled plaque DNA from one individual (plaque a,b,c and d) as shown in table 5.1. The transfection efficiency (number of foci/μg DNA) for plaque DNA ranged from 0.025 to 0.133 foci/μg DNA (mean 0.070 foci/μg DNA). No foci arose in cells transfected with normal aorta, umbilical cord artery or C3H/10T1/2

Tabel 5.1 Transforming activity of DNA from human atherosclerotic lesions and controls.

Origin	foci/total plates	foci/μg DNA
Primary transfections		
plaque A	3/9	0.025
plaque B	8/3	0.138
plaque C	3/2	0.075
plaque a	4/5	0.040
plaque b	2/2	0.050
plaque c	9/5	0.090
plaque d	8/5	0.080
overall primary transfections		0.071
normal aorta	0/15	≤0.003
umbilical cord artery	0/18	≤0.003
C3H/10T1/2 cells	0/4	≤0.013
MCA-treated cells	5/2	0.125
Secondary transfections		
focus 1	7/5	0.070
focus 2	19/9	0.106
focus 3	8/3	0.080
focus 4	18/3	0.300
focus 5	11/5	0.110
focus 6	29/9	0.161
focus 7	65/5	0.650
overall secondary transfections		0.211

cell DNA indicating the absence of spontaneous cell transformation. DNA derived from MCA-induced (primary) transformants was used as a positive control and resulted in a transfection efficiency of 0.125 foci/μg DNA. Plaque DNA-induced foci and MCA-induced transformants were visible as closely packed and highly overlapping disorganized polar spindle cells. Plaque-induced foci cells were isolated and grown in mass culture for the isolation of DNA and used in a second round of transfection. The efficiency of secondary transfection ranged from 0.070 to 0.650 foci/μg DNA (mean 0.211 foci/μg DNA) showing that the transforming elements in plaque DNA can be transmitted serially with a transfection efficiency increased about three-fold as compared with the first cycle of transfection. The cells transformed by plaque DNA and their secondary transformants gave rise to tumors after injection into nude mice (table 5.2) indicating that these cells possess tumorigenicity *in vivo*. All tumors derived from either plaque-DNA-induced transformants or MCA-induced transformants, were morphologically similar (Fig 5.1).
To examine whether the transformed C3H/10T1/2 mouse cells contain human plaque DNA sequences hybridization experiments were performed using as a probe the human

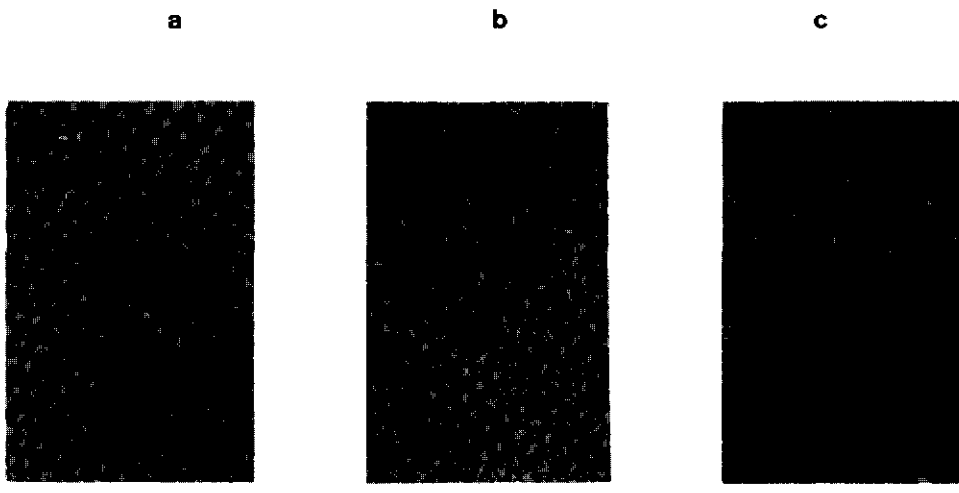

Fig. 5.1 Photographs of C3H/10T1/2 cells in cell culture (a,b) and grown after injection in nude mice (c). a. Cell culture of C3H/10T1/2 cells after transfection with C3H/10T1/2 cell DNA. b. Transformed cells inside a focus type III after transfection with human plaque DNA. c. Histological section (Hematoxylin and Eosin stained) of a tumor, arisen after injection of (secondary) transformants.

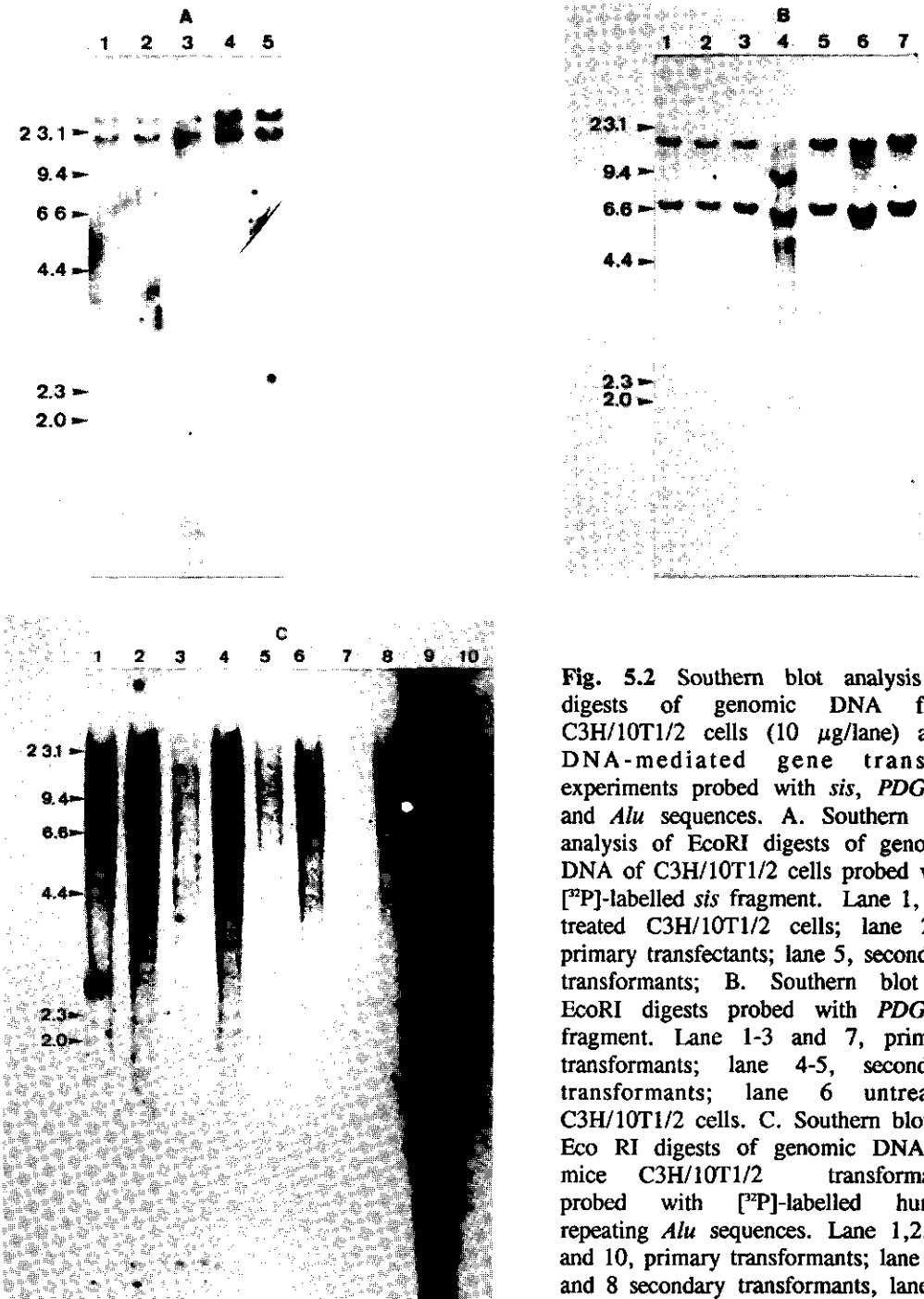

Fig. 5.2 Southern blot analysis of digests of genomic DNA from C3H/10T1/2 cells (10 μg/lane) after DNA-mediated gene transfer experiments probed with *sis*, *PDGF-A* and *Alu* sequences. A. Southern blot analysis of EcoRI digests of genomic DNA of C3H/10T1/2 cells probed with [^{32}P]-labelled *sis* fragment. Lane 1, untreated C3H/10T1/2 cells; lane 2-4, primary transfectants; lane 5, secondary transformants; B. Southern blot of EcoRI digests probed with *PDGF-A* fragment. Lane 1-3 and 7, primary transformants; lane 4-5, secondary transformants; lane 6 untreated C3H/10T1/2 cells. C. Southern blot of Eco RI digests of genomic DNA of mice C3H/10T1/2 transformants probed with [^{32}P]-labelled human repeating *Alu* sequences. Lane 1,2,4,6 and 10, primary transformants; lane 3,5 and 8 secondary transformants, lane 9, normal human aorta (0.2 μg) and lane 7 control C3H/10T1/2 cells. The sizes of the DNA markers on the left are given in kb.

repetitive *Alu* sequence, a 300 bp Bam HI insert of pBLUR8. DNA from primary foci cells displays a strong hybridization signal over a wide size range of DNA fragments (Fig. 5.2). The DNA of normal human aorta and C3H/10T1/2 cells shows positive and no hybridization signal with the *Alu* probe, respectively (Fig. 5.2). Likewise, *Alu* positive signals were present in DNA of secondary transformants (Fig. 5.2) indicating that all transformed cells contain human DNA sequences.

Tabel 5.2 Tumorigenicity of (transformed) C3H/10T1/2 cells

Origin cells	number of mice	number of tumors
C3H/10T1/2 cells	15	0
primary transformants	25	6
secondary transformants	22	9
MCA-induced transformants	6	2

For the identification of transforming genes cDNAs encoding several known oncogenes, as *sis*, *PDGF-A*, *mos*, *fos*, *myc*, *H-ras*, *Ki-ras* and *N-ras* were used in hybridization experiments with DNA derived from primary and secondary transformants. Hybridization patterns of DNAs from (primary as well as secondary) transformants with all the probes, excluding PDGF-A, did not differ significantly from those of C3H/10T1/2 cells. DNA after Eco RI digestion hybridizing with a PDGF-A probe shows an extra band of 9.2 kb, besides the endogenous molecular weight bands of 16.6 and 6.9 kb of the C3H/10T1/2 cells in only one of the seven primary and the associated secondary transformed cell lines (Fig. 5.2). BamH I digests of genomic DNA of these transformants resulted also in extra PDGF-A positive DNA fragments (results not shown). This proves that at least in some instances the transforming nature of plaque DNA can be attributed to the presence of the human PDGF A gene.

DISCUSSION

This report clearly shows the transforming potential of DNA from human atherosclerotic lesions. The human (*Alu* sequences containing) transforming elements can be transmitted serially and in an *in vivo* assay for tumorigenicity give rise to tumors in athymic mice. This provides strong evidence that these lesions are the direct results of activities of transforming genes present in fibrous plaques. This finding has important implications for the understanding of molecular events taking place in the proliferation of smooth muscle cells and the development of atherosclerosis. In particular, they provide a possible explanation for the observations of Benditt and Benditt (1973) that atherosclerotic lesions undergo monoclonal cell proliferation. In addition, we have shown that in one of the seven primary transformants tested a PDGF-A gene is probably responsible for the completion of cell transformation. In the other transformants no extra PDGF-A positive DNA fragments were seen. So, this indicates that several transforming genes may be involved in atherogenesis and the PDGF-A gene being one of them. These data are in agreement with the hypothesis that regulation of smooth muscle cell proliferation in atherosclerosis is multifactorial.

The present study shows that DNA derived from aortic lesions has transforming potential in C3H/10T1/2 cells, as has DNA from coronary lesions in NIH 3T3 cells (Penn et al., 1986; Ahmed et al., 1990). Moreover, cultured cells derived from human atherosclerotic lesions also possess transforming genes (Parkes et al., 1991). This would indicate that a transforming potential detectable in different cell systems is a general feature of plaque DNAs from different origins. This is in striking contrast with the study of Yew et al. (1989), who failed to find transforming activity of human plaque DNA. Because all these studies (including this study) were performed with *pooled* samples of DNA transforming genes possibly only occur in a subset of the human plaques. So, our results emphasize the contribution of genetical alterations in atherogenesis, but do not exclude other potential mechanisms critical to atherogenesis, such as paracrine growth factor models.

Our observation that PDGF-A is a transforming gene in human plaque DNA provides support for the involvement of an autocrine PDGF-A cycle in smooth muscle cell proliferation. PDGF is a basic protein with two disulphide-linked chains, designed as A and B, which can form homodimers as well as AB heterodimers. Both chains are

produced by macrophages and endothelial cells, and only the A-chain is secreted by smooth muscle cells in tissue culture. (Martinet et al., 1986; Sejersen et al., 1986; Starksen et al., 1987; Ross, 1986/1990). The genes are located on different chromosomes and both possess transforming capacities; the gene encoding the human PDGF-B chain is the normal counterpart of the *v-sis* oncogene (Waterfield et al., 1983; Beckmann et al., 1988). Detection of enhanced transcription levels of the PDGF-A and PDGF-B genes in atherosclerotic lesions as compared with normal arteries indicates that these genes could play a role in atherosclerosis (Barrett and Benditt, 1987; Libby et al., 1988; Wilcox et al., 1988). Increased concentrations of PDGF-A transcripts have been localized to cells inside human atherosclerotic lesions, whereas B chain transcripts colocalized primarily with endothelial cells and macrophages (Wilcox et al., 1988; Libby et al., 1988). This implicates that plaque cells possess the capacity to produce an endogenous, potentially self-stimulatory (autocrine) growth factor. Moreover, PDGF-A gene expression in human smooth muscle cells can be induced by atherogens, like (oxidized) low density lipoproteins (Zwijsen et al., 1992). These data emphasize the role of PDGF-A in atherogenesis and illustrate the interesting parallel between neoplasia and atherosclerosis. Otherwise, the present study failed to demonstrate that the *sis* gene is an active transforming gene present in human plaque DNA and therefore PDGF-B might play a role in atherogenesis as paracrine proliferation mechanism.

The question remains which other transforming genes are involved in the development of atherosclerosis. The present study indicates that *mos*, *myc*, *Ha-ras*, *Ki-ras*, *N-ras* and *fos* are to be excluded.

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CHAPTER 6

CELL TRANSFORMING POTENTIAL OF LOW DENSITY LIPOPROTEINS

Arterial smooth muscle cell proliferation is thought to be essential for the development of atherosclerotic lesions. The monoclonal hypothesis of atherogenesis proposes that the proliferative smooth muscle cells are derived from a stable transformed cell population. The present study deliveres for the first time evidence that, except carcinogens as 3-methylcholantrene (MCA), also 'atherogenic' low density lipoproteins (LDL) possess cell transforming potential. LDL caused dose-dependent cytotoxicity and transformation of C3H/10T1/2 cells of type II and type III morphology up to 6 transformed cell clones per 10^4 survivors in the concentration range of 50-200 μg cholesterol/ml after 72 h treatments. MCA (0.1 μg/ml) induced morphological transformation of 2.6 foci per 10^4 survivors. In a two stage *in vitro* transformation assay LDL (5-25 μg cholesterol/ml) enhanced MCA-induced cell transformation 2-2.4 fold in a dose-dependent way. 'Non-atherogenic' high density lipoproteins (HDL) did not induce celtransformation by themselves or in an initiation-promotion model. These results show that LDL could act as (co)carcinogens.

This chapter is based on:

Renate M.L. Zwijsen (1992) Cell transforming potential of low density lipoproteins. Carcinogenesis 13, 1913-1915.

INTRODUCTION

Proliferation of smooth muscle cells in the intima of arterial vessels has been viewed as a key mechanism in atherosclerosis. It has been suggested that the proliferative smooth muscle cells are derived from a stable transformed cell population (Benditt and Benditt, 1973). The link between atherogenesis and carcinogenesis is supported by (a) the monoclonal character of lesion cells (Benditt and Benditt, 1973), (b) the presence of transforming genes in atherosclerotic lesions (Penn et al., 1986; Ahmed et al., 1990) and (c) the induction of lesions by carcinogens (Albert et al., 1975; Bond et al., 1981; Albert et al., 1977; Paigen et al., 1985), radiation (Gold, 1961) and oncogenic viruses (Fabricant et al., 1978; Minick et al., 1979). Moreover, atherosclerotic lesions could be induced after a sequential treatment of 7,12-dimethylbenz[a]anthracene and methoxamine administrated in an initiation-promotion protocol in cockerels (Majeski et al., 1985).

However, the cell transforming activities of known atherogens have never been investigated. Consequently, the present investigation was undertaken to clarify whether lipoproteins as low density lipoproteins (LDL) and high density lipoproteins (HDL) possess promoting and transforming capacities using C3H/10T1/2 cells. The C3H/10T1/2 fibroblast cell line has been used as a model system to study oncogenic transformation by chemical carcinogens themselves (Reznikoff et al., 1973). The transformation of C3H/10T1/2 cells can be observed to proceed through discrete stages of initiation and promotion comparable with *in vivo* transformation (Frazelle et al., 1983; Boreiko, 1985). This has permitted use of this cell transformation system for the study of agents that transform cells, initiate transformation and enhance (promote) transformation in an initiation-promotion sequence protocol. We used 3-methylcholantrene (MCA) as an initiator and 12-0-tetradecanoylphorbol-13-acetate (TPA) or lipoproteins as promoters.

MATERIALS AND METHODS

Testcompounds used were purchased from Sigma, St. Louis, MO. The lipoproteins LDL and HDL were isolated from fresh unfrozen human plasma (Sigma) and were checked for their purity as described previously (Zwijsen et al., 1990).

For the cell transformation tests C3H/10T1/2 clone 8 cells (passage 11-13) were cultured in Eagle's Minimal Essential Medium (EMEM) with 10% fetal calf serum (FCS) and gentamycin (50 μg/ml). 4-5 10^4 cells were plated in 6 well-plates (Costar) used for cell transformation tests and diluted to 300 cells/well for plating efficiency tests to determine cytotoxicity. Cells were exposed for 72 h to test compounds in EMEM without serum followed by culturing in EMEM supplemented with 10% FCS. In initiation-promotion experiments 6 days after plating cells were secondly exposed to the test compounds TPA, LDL or HDL in EMEM with 10% lipoprotein deficient serum (LPDS) for two weeks. Culture medium was refreshed twice weekly. Control cell cultures without exposure to the test compounds were grown in the same medium as treated cells. Five days and five weeks after culturing for plating efficiency tests and celtransformation tests, respectively, experiments were terminated by fixation with methanol/acetic acid (3:1) followed by 5% (v/v) Giemsa staining. The number of cell clones and the total number of foci (type II or III as defined by Reznikoff et al. 1973) were scored blind. Twelve wells were used in each treatment group and two independent tests were done.

RESULTS AND DISCUSSION

Data of the 'two stage *in vitro* transformation' protocol are summarized in Table 6.1. These tests were performed at non-cytotoxic concentrations and untreated C3H/10T1/2 cells possessed a plating effiency of 17 $\pm$ 1%. Treatment with the carcinogenic agens MCA (0.1 μg/ml) during the 'initiation' period of three days resulted in 2.6 transformed cell clones (foci) per 10^4 cells. Additional exposure of LDL (5-25 μg cholesterol/ml) to MCA-treated cells in the 'promotion' period resulted in enhancement of transformation of type II and type III morphology with a factor 2 - 2.4, which was dose-dependent. HDL did not modulate the number of transformed clones induced by MCA. Exposure of (sub)confluent cells during the 'promotion' period without MCA pretreatment to LDL or HDL did not gave rise to transformed cells. So, the present study using a two stage MCA-induced transformation model clearly demonstrated promoting activities of LDL. These data corroborate existing data, which demonstrated that atherogens like LDL reduced gap junctional communication, a mechanism which could result in promotion of

Table 6.1 Two stage *in vitro* transformation of C3H/10T1/2 cells[a].

Initiator (μg/ml)	Promotor (μg/ml)	No. of type II and type III/total No. of wells experiment 1	experiment 2	Transforming frequency/ 10^4 survivors
-	-	0+0/12	0+0/12	0.0
MCA[b]				
0.1	-	26+17/12	28+18/12	2.6
	TPA			
-	0.1	0+0/12	0+0/12	0.0
0.1	0.1	287+207/12	313+150/12	35.8
	LDL[c]			
0.1	5	52+40/12	41+48/12	5.3
0.1	10	53+36/11	45+51/52	5.6
0.1	25	47+56/12	65+42/12	6.2
-	5	0+0/10	0+0/12	0.0
-	10	1+0/12	0+0/12	0.1
-	25	1+1/10	2+1/11	0.2
	HDL[c]			
0.1	5	22+12/12	19+28/12	2.7
0.1	10	19+23/12	23+19/12	2.5
0.1	25	22+9/12	27+17/12	2.2
-	5	0+0/12	0+0/12	0.0
-	10	0+0/12	0+0/12	0.0
-	25	1+0/12	0+0/12	0.1

[a]Plating efficiency of untreated cells was 17% (range 14% to 20%); absolute no. of clones was 49.8.
[b]Relative cloning efficiency of MCA treated cells was 99% (range 92% to 103%).
[c]Lipoproteins are expressed as μg cholesterol/ml.

(abnormal) smooth muscle growth (Zwijsen et al., 1990; Zwijsen et al., 1991).

Data of the transforming capacity of MCA, LDL and HDL are presented in Table 6.2. MCA served as a positive control (3.6 type II and type III foci/well). LDL treatment (50 μg cholesterol/ml - 200 μg cholesterol/ml) for three days led to induction of both type II and type III foci (1.1 - 1.3 type II or type III foci/well). As these concentrations are cytotoxic in a dose-dependent manner the transforming frequency per 10^4 survivors ranged

between 0.8 at 50 μg LDL-cholesterol/ml to 4.1 at 200 μg LDL cholesterol/ml. Treatment with HDL over the same concentration range as LDL did not result in any transformation of C3H/10T1/2 cells. So, of these two lipoproteins, only LDL possessed transforming potential over C3H/10T1/2 cells.

Table 6.2 Transforming potential of MCA, LDL and HDL.

Treatment (μg/ml)	Survival[a] (%)	No. of type II and type III/ total No. of wells		Transforming frequency/ 10^4 survivors
		experiment 1	experiment 2	
-	100	0+0/12	0+0/12	0.0
MCA				
0.1	98	24+12/12	25+25/12	2.5
LDL[b]				
50	91	4+5/10	10+5/12	0.8
100	71	10+6/12	7+8/12	1.2
150	24	9+6/12	12+5/12	3.6
200	18	8+7/12	6+5/12	4.1
HDL[b]				
50	98	0+0/12	0+0/12	0.0
100	102	0+0/12	0+0/12	0.0
150	94	0+0/12	0+0/12	0.0
200	97	2+0/12	0+0/11	0.1

[a]Plating efficiency of untreated cells was 17% (range 12% to 21%); absolute no. of clones was 51.0.
[b]Lipoproteins are expressed as μg cholesterol/ml.

C3H/10T1/2 cells are immortalized, partially transformed mouse fibroblast cell lines. The present study shows that treatment with LDL was sufficient to transform C3H/10T1/2 cells fully. This indicates that LDL is capable of activating transforming gene(s) leading to cell transformation. Recently, it was demonstrated via gene transfer procedures that human plaque DNA was capable of transforming NIH 3T3 cells in culture. In a concommitant *in vivo* assay for tumorigenicity these transformants give rise to tumors in athymic mice (Penn et al., 1986; Ahmed et al., 1990). If the transforming gene(s) present

in atherosclerotic lesions is identified it will become possible to study how specific (environmental) agents as LDL interact with this gene. In conclusion, the results of the present investigation indicate that the 'atherogenic' LDL possesses cell transforming potential and clearly shows that LDL has promoting activities in an initiation-promotion model. In view of these findings atherogens may behave like carcinogens with regard to their transforming potency. These findings are consistent with the hypothesis that the intimal proliferating cells present in atherosclerotic lesions are transformed cells (Benditt and Benditt, 1973). However, further work is needed to demonstrate whether atherosclerotic lesions can be considered as neoplasms.

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CHAPTER 7

INDUCTION OF PLATELET-DERIVED GROWTH FACTOR CHAIN A GENE EXPRESSION IN HUMAN SMOOTH MUSCLE CELLS BY OXIDIZED LOW DENSITY LIPOPROTEINS

Abnormal proliferation of vascular smooth muscle cells is a key event in the formation of atherosclerotic plaques in humans. It has been suggested that modulation of platelet derived growth factor chain A (PDGF-A) gene expression may contribute to atherosclerosis. Using an '*in situ* hybridization' technique the present study shows that the presence of low density lipoproteins (LDL) induces an eight-fold increased expression of the transforming gene PDGF-A in human smooth muscle cells. The influence on PDGF-A expression only occurred after oxidative modification of LDL, a process known to be important in atherogenesis. The non-atherogenic high density lipoproteins (HDL) did not modulate the PDGF-A mRNA levels in smooth muscle cells. Consequently, oxidized LDL may participate in atherosclerotic lesion formation as a result of autocrine stimulation of PDGF-A.

This chapter is based on:

Renate M.L. Zwijsen, Sandra C. Japenga, Astrid M.P. Heijen, Rommert C. van den Bos and Jan H. Koeman (1992) Induction of platelet-derived growth factor chain A gene expression in human smooth muscle cells by oxidized low density lipoproteins. Biochem. Biophys. Res. Commun. 186, 1410-1416.

INTRODUCTION

Atherosclerosis is characterized by a nodular proliferation of smooth muscle cells in the arterial intima. Platelet-derived growth factor (PDGF) has been hypothesized to be responsible for this intimal proliferation in atherogenesis. PDGF is composed of two polypeptide chains (A and B) encoded by separate genes (Heldin et al., 1979; Betsholtz et al., 1986). The subunits can form dimers in different combinations to create biologically active PDGF. Macrophages as well as endothelial cells synthesize both chains of PDGF, whereas smooth muscle cells appear to be activated to express only PDGF-A (Martinet et al., 1986; Sejersen et al., 1986; Starksen et al., 1987). Recent studies demonstrated that PDGF-A transcripts can be localized in intimal lesion cells inside human atherosclerotic lesions suggesting an important role of the PDGF-A gene in atherogenesis (Wilcox et al., 1988; Libby et al., 1988).

The observed modulation of PDGF-A expression in atherosclerotic lesions might be induced by known atherogens. Factors strongly correlated with atherogenesis are high plasma levels of Low Density Lipoproteins (LDL) and low plasma levels of High Density Lipoproteins (HDL). There is increasing evidence indicating that atherogenicity of LDL is enhanced by oxidative modification (Morel et al., 1984; Steinberg et al., 1989). Large amounts of oxidized LDL were shown to be present in atherosclerotic lesions (Haberland et al., 1988; Palinsky et al., 1989). However, it is unclear whether such (oxidized) LDL stimulates growth factor gene expression. The purpose of this study is to investigate the influence of oxidized and native LDL as well as HDL on PDGF-A expression in human smooth muscle cells. The present study is the first report on the modulation of PDGF chain A expression by LDL using an *in situ* hybridization technique. LDL appeared to be effective only after oxidative modification.

MATERIALS AND METHODS

Chemicals

Salmon sperm DNA (ssDNA), bovine serum albumin (BSA), LDL and HDL were purchased from Sigma (St. Louis, MO). The lipoproteins were checked for purity as

described previously (Zwijsen et al., 1990). ^{3}H-uridine, ^{3}H-desoxyadenosine triphosphate and ^{3}H-thymidine were from Amersham (Buckinghamshire, UK), butylated hydroxytoluene (BHT) and formamide were from Merck (Darmstadt, Germany). Total yeast RNA and vanadylsulphate were obtained from BDH (Poole, England).

Probes

As a probe specific for the PDGF A chain a 1.3 kb Eco RI fragment of a cDNA, D1 (Betsholtz et al., 1986) labeled by random priming (Feinberg and Vogelstein, 1983) was used. To check for aspecific binding cells were hybridized with pBR322 fragments. Both probes were labeled to a specific radio activity of about 10^9 cpm/μg DNA.

Smooth muscle cell culture and exposure.

Smooth muscle cells were isolated from arteries of human umbilical cords. After removal of the adventitial layer one mm^2 intimal-medial explants were placed on 18 x 18 mm glass cover slips. The explants were incubated in EMEM (Flow Laboratories, U.K.) supplemented with 5% FCS (Gibco, UK), 5% human serum (Sigma, St. Louis, MO) and the antibiotics gentamycin (50 μg/ml) and amphotericin B (1.5 μg/ml) in a 37 °C, 5% CO_2 humidified air environment. After 2 -3 weeks cells derived from the peripheral growth zone were subcultured in flasks (Costar) containing EMEM, 10% FCS and the same antibiotics. Distinction between smooth muscle cells, fibroblasts and endothelial cells was made by means of fluoresceinated antibodies specific against smooth muscle actin (Chamley et al., 1977) and the growth pattern of smooth muscle cells in culture ("hills and valleys"). All experiments were performed with smooth muscle cell cultures of passage 2 through 3 free of fibroblasts and endothelial cells.

1 x 10^4 Smooth muscle cells were plated on cover slips at 16 h before treatment. The cells were exposed to LDL or HDL for different periods of time and concentrations in F10 medium (Flow Laboratories, U.K.) containing 10% lipoprotein deficient serum (LPDS, Sigma, St Louis, MO) and antibiotics. To study the role of oxidative modification of LDL on PDGF-A expression, cells were treated with LDL (50-200 μg cholesterol/ml) in the presence of BHT (20 μM) for 8 h. Control cultures were exposed to F10 medium and LPDS without test compounds.

In situ hybridization

Cells were plated on 18x18 mm cover slips coated with 0.5% gelatin. After treatment cells were fixed in 4% (w/v) paraformaldehyde and acetylated with 0.1 M triethanolamine (pH 8.0) mixed with 0.25% (w/v) acetaldehydanhydride for 10 min. Cells were washed with 2xSSC (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate pH 7.0) and then treated with a solution of 0.1M glycine, 0.2 M Tris.HCl pH 7.4 for 10 min. Prehybridization was performed in 50% (v/v) deionized formamide/2xSSC for 10 min and hybridization was done in the same solution after addition of 1% (w/v) BSA, 10mM vanadylsulphate, 10% (w/v) dextransulphate, 0.5 mg/ml ssDNA, 2 mg/ml total yeast RNA and 1.2 ng/μl ^{3}H labeled probe (9 ng/coverslip). After hybridization for 20-24 h at 37 °C cover slips were washed in 2xSSC/ 50% (v/v) formamide at 37 °C, 1xSSC/50% (v/v) formamide at 37 °C followed by 1xSSC at room temperature, each during 10 minutes. The glasses were processed for autoradiography using NTB-2 nuclear emulsion (Kodak Co) and exposed in the dark at 4 °C for 7 days. After development cells were counterstained with toluidin blue. All experiments were carried out in duplicate and for each treatment three cover slips were prepared. On each slip 25 cells were scored for the number of silver grains. Hybridization levels of a specific mRNA transcript were determined from the number of grains per cell after specific hybridization. Background hybridizations were estimated from the number of grains per cell after hybridizations with pBR322 as a non-specific probe.

Total RNA assay

The amount of total cellular RNA in smooth muscle cells was determined to ensure comparability. 1 x 10^5 Cells were plated into 24-wells tissue culture plates (Costar). After attachment and (in several cases treatment) cells were exposed to ^{3}H-uridine (10 μCi/ml) for 2 h at 37 °C. Cells were washed with PBS and exposed to 5% (w/v) trichloroacetic acid (0 °C) to precipitate RNA. Cells were washed with 0.5% (v/v) Triton in 0.1 M phosphate buffer (pH 7.5), scraped from the bottom and sonicated for 5 min. After centrifugation the radioactivity in the supernatant was measured. Each experiment was done in four-fold and at least two independent experiments were performed.

Statistical analysis

The significance of different treatments on PDGF-A gene expression and the amounts of total cellular RNA in smooth muscle cells compared with untreated cells were tested with a Student t-test.

RESULTS

Human smooth muscle cells were treated with LDL or HDL followed by hybridization with a probe specific for PDGF-A. The influence of different lipoproteins on PDGF-A expression in human smooth muscle cells is shown in Fig. 7.1. The values are corrected for aspecific hybridization; *in situ* hybridization with ^{3}H labeled pBR322 gave rise to 0.33 $\pm$ 0.13 grains/cell. Smooth muscle cells contained 0.5-0.6 PDGF-A transcripts/cell under culture conditions. Exposure to LDL (100 μg cholesterol/ml) caused a 4 - 8 fold increase in PDGF-A transcripts. The maximal level was found after 8 h of LDL-incubation; under these conditions four copies of the mRNA per cell were detected. After 14 h exposure to LDL the PDGF-A transcript level diminished to two copies/cell; this value remained

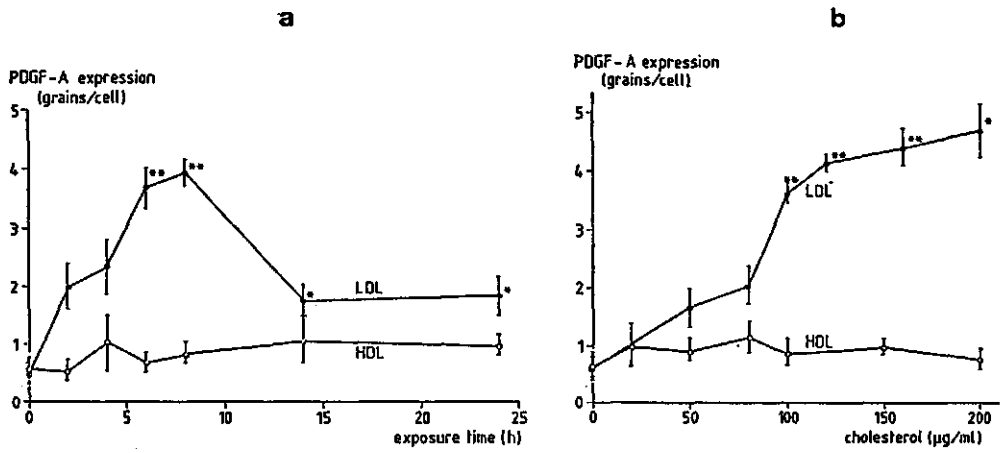

Fig. 7.1 Quantification of PDGF-A transcript levels in human smooth muscle cells using the '*in situ* hybridization' technique after exposure to LDL or HDL for different periods at 100 μg cholesterol/ml (a) and to different concentrations for 8 h. (b). Values are mean number of grains per cell $\pm$ SEM. Asterisks indicate significant differences from untreated cells ($^*p \leq 0.05$; $^{**}p \leq 0.01$).

stable for periods up to 24 h. As shown in Fig. 7.1a HDL did not influence the PDGF-A transcript level. After 8 h of LDL treatment the levels were maximal and therefore after this period the PDGF-A expression levels were determinated for several LDL and HDL concentrations. Fig. 7.1b demonstrates the LDL dose-dependent transcript levels. Exposure to LDL (200 μg cholesterol/ml) resulted in 4.7 PDGF-A transcripts/cell compared with 0.6 in cultures of untreated smooth muscle cells. HDL treatment (50 - 200 μg cholesterol/ml) did not influence PDGF-A expression levels in smooth muscle cells.

Table 7.1 Quantification of PDGF-A chain transcript levels in human smooth muscle cells using the '*in situ* hybridization' technique after exposure to low density lipoproteins (LDL) in the presence or absence of 20 μM butylated hydroxytoluene (BHT). Data are expressed as mean number of grains per cell ± S.E.M.

LDL (μg cholesterol/ml)	BHT -	BHT +
0	0.52 ± 0.21	0.71 ± 0.30
50	1.64* ± 0.52	0.78 ± 0.31
100	3.16** ± 0.39	0.94 ± 0.25
200	3.93** ± 0.54	0.60 ± 0.19

* significantly different from the control; $p \leq 0.05$

** significantly different from the control; $p \leq 0.01$

To examine the role of oxidative modification of LDL in the LDL-induced gene expression cells were incubated with both LDL and the antioxidant BHT (20 μM) for 8 h. Table 7.1 shows that incubation of smooth muscle cells with LDL (50 - 200 μg cholesterol/ml) in the presence of BHT (20 μM) caused no significant increase in the number of PDGF-A mRNAs as compared to untreated cells. Thus, BHT appears to prevent the LDL-induced increase of PDGF-A expression. Therefore, oxidative modification of LDL is required for modulating PDGF chain A transcript levels.

Total RNA levels were determined with a ^{3}H-uridine incorporation assay. The means of the RNA levels per cell after exposure to LDL or HDL were between 95% and 115% of

control values. Therefore, the observed PDGF-A mRNA modulation could not be ascribed to changes in total RNA content per cell.

DISCUSSION

The present study shows that LDL in contrast to HDL markedly increases PDGF-A transcript levels in human smooth muscle cells and that only the oxidized form of LDL plays an active role in this context. This observation is consistent with a number of studies indicating that the antioxidant BHT causes a blockage of several LDL-modulated effects (Morel et al., 1984; Steinberg et al., 1989; Zwijsen et al., 1991). LDL oxidation may occur *in vivo* and *in vitro* and is completely inhibited by BHT (Haberland et al., 1988; Palinsky et al., 1989; Morel et al., 1984; Steinberg et al., 1989; Zwijsen et al., 1991). The fact that antioxidant therapy slows the progress of atherosclerotic lesions has provided further evidence that oxidized LDL does play a pathogenetic role in atherogenesis (Carew et al., 1987).

PDGF A gene products possess cell transforming capacities and expression of this gene has been found in several sarcoma's (Heldin et al., 1986; Beckman et al., 1988; Rorsman et al., 1988). Besides in carcinogenesis, PDGF-A might play an important role in atherogenesis. The present report shows that oxidized LDL increased PDGF-A expression *in vitro*. The suggestion that this process may play a role in atherogenesis is strenghtened by the observation that both oxidized LDL and increased PDGF-A expression were detected in atherosclerotic lesions (Wilcox et al., 1988; Libby et al., 1988; Haberland et al., 1988; Palinsky et al., 1989). Moreover, the intimal cells of human atherosclerotic plaques were both able to produce PDGF and respond to PDGF (Libby et al., 1988). So, oxidized LDL may participate in atherogenesis due to autocrine stimulation of PDGF-A. Besides by oxidized LDL, expression of the PDGF A gene could be increased by several other substances, including phorbol esters, thrombin and transforming growth factor ß (Starksen et al., 1987).

In this report gene expression in human atheroma was examined by an '*in situ* hybridization' technique in stead of the generally used method based on the analysis of total RNA extracts from cell cultures (Chirgwin et al., 1979; Chomczynski and Sacchi,

1987). The approach based on total RNA extracts is useful for evaluating the capacity of a defined cell population, such as cell lines, to express a specific gene. However, determination of the effects of several treatments on transcript levels in (primary) cell cultures demands large numbers of cells. In addition, quantitative comparisons between cell isolates subjected to passages *in vitro* may reflect not only the treatments of the cells, but also differences in donors, passage levels and cell densities. The *in situ* technique is a sensitive, powerful method for defining (relative) transcript levels in a few cells of the same origin. So, this method offers exceptional opportunities to examine the influence of various compounds on the expression of genes in individual cells.

However, mRNA studies do not prove unequivocally that these cells actually translate this message or process it into an active protein product. Furthermore, we cannot rule out the existence of variants in biologically active chain A polypeptides, because this can not be detected with the *in situ* hybridization approach used here (Rorsman et al., 1988).

Benditt proposed that atherosclerosis represents a monoclonal, neoplastic smooth muscle cell growth in the intima of arteries (Benditt and Benditt, 1973). This hypothesis was supported by the findings that infection with a herpes virus (Marek's disease), radiation and carcinogens can produce atherosclerotic lesions (Minick et al., 1979; Gold, 1961; Albert et al., 1975). Even focal smooth muscle cell proliferation could be induced after a sequential exposure to 7,12-dimethylbenzo(a)anthracene and methoxamine administrated in an initiation-promotion protocol (Majesky et al., 1985). Moreover, transforming genes were isolated from human coronary artery plaques (Penn et al., 1986; Ahmed et al., 1990). The findings of the present study also illustrate the parallel between neoplasma and atheroma and support the hypothesis that smooth muscle cells possess the capacity to produce an endogenous, potentially self stimulatory (autocrine) growth factor, namely PDGF-A.

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CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

The principal lesion characteristic of atherosclerosis is the plaque. This lesion mainly consists of smooth muscle cells, connective matrix and large amounts of extracellular lipids. Smooth muscle cell hyperplasia is an integral event in atherosclerotic plaque formation and the resultant occlusion of blood vessels. These abnormal cell proliferations are primarly caused by defects in the autocrine and/or paracrine growth regulation. A novel mechanism critical to atherogenesis introduced in the present study is gap junctional mediated growth control. The atherogen-induced effects on gap junctional communication between human smooth muscle cells are described in chapters 2,3 and 4. The role of autocrine growth stimulation in atherogenesis is shown by the demonstration of the presence of activated transforming genes in atherosclerotic lesions (chapter 5) and the cell transforming potential of (atherogenic) low density lipoproteins (LDL) as shown in chapter 6. Chapter 5 also demonstrated that several transforming genes may be present in human plaque DNA, one of them being PDGF-A (platelet-derived growth factor chain A). The expression of the latter gene can be modulated by oxidized LDL (chapter 7).

The present investigation provides additional evidence for the hypothesis that autocrine production of growth modulating factors may contribute to the characteristic abnormal smooth muscle growth in atherogenesis. We demonstrated that lipoproteins with atherogenic potential, such as LDL, in contrast to the non-atherogenic HDL, are able to transform fibroblasts *in vitro*. Genetical alteration of intimal cells present in atherosclerotic lesions was demonstrated using DNA-mediated transfection techniques. It could be demonstrated that human aortic plaque DNA contains active transforming genes, as PDGF-A. This is in agreement with studies by Penn et al. (1986) and Ahmed et al. (1990), showing transforming genes in human coronary artery plaques. Even DNA of cultured human atherosclerotic plaque cells appeared to have cell transforming capacities (Parkes et al., 1991). Only Yew et al. (1989) could not demonstrate a transforming potential of human plaque DNA. The variable results obtained sofar might indicate that different mechanisms are involved. It should be stressed in this respect that in all these studies pooled plaque samples were used and it should be realized that the transforming potential is not necessarily present in each plaque. The observed transforming potential of plaque DNA supports very well the monoclonal hypothesis of Benditt and Benditt (1973). They observed that atherosclerotic lesions start as singular focal masses containing

monoclonality and suggested that these masses were in general similar to benign tumors as observed in other tissues. However, it has been argued that apparent monoclonality could also arise because of strong selection pressure favoring a subpopulation of cells, although findings of transforming genes, such as PDGF-A, present in atherosclerotic lesions and the transforming potential of atherogens indicate the opposite. Additional arguments in favor of the monoclonal hypothesis are that tumor initiators like chemical mutagens and promutagens (Albert et al., 1975; Bond et al., 1981), radiation (Gold, 1961) and oncogenic viruses (Minick et al., 1979) can induce atherosclerotic lesions in laboratory animals. Hence, molecular alterations underlying the proliferation of smooth muscle cells could show resemblance to the molecular events, which are critical in the development of cancer.

The current view on atherosclerosis is based on the "response to injury" hypothesis, implying a paracrine stimulation of smooth muscle growth as a result of injury of endothelium (Ross, et al., 1976; Ross, 1981). Most of the studies of experimental atherogenesis are implemented in the framework with this hypothetical model. Support for this hypothesis is based on the following observations. Atherosclerotic lesions can be induced in experimental animals by endothelial denudation and factors derived from platelets can induce smooth muscle growth *in vitro*. Injury to arterial endothelium by mechanical, chemical, toxic, viral, or immunological agents caused endothelial denudation, and was followed by platelet adhesion and aggregation, with consequent release of platelet-derived growth factors (PDGF), in turn leading to migration into and proliferation of smooth muscle cell in the intima and secretion of connective tissue components. Over the years, the hypothesis has been modified (Ross, 1990). First of all, it became apparent that actual denudation was not a consistent early feature of atherosclerosis (Davies et al., 1976). Secondly, platelet adherence is neither necessary nor sufficient to cause the lesions (Schwartz and Reidy, 1987). Presently, the view is: a. the endothelium can respond to a variety of stimuli, by subtle changes in function (endothelial dysfunction) and/or by the induction of new endothelium properties; thus non-denuding injury may be important in initiating the lesions of atherosclerosis and b. platelets are not the sole initiators of smooth muscle proliferation, since growth promoting and growth inhibitory factors secreted by other cell types, including macrophages and endothelial cells, may modulate smooth muscle growth.

However, not all observations made can be explained by the "response to injury" hypothesis, including the findings that (intimal) smooth muscle cells themselves can secrete and can respond to growth modulating factors and the cell transforming potential of plaque DNA as demonstrated above, thus implying an involvement of autocrine growth stimuli. A good candidate for autocrine growth factor stimulation of smooth muscle cells is PDGF-A. High transcript levels of this transforming gene were detected in intimal cells of human atherosclerotic lesions (Libby et al., 1988; Wilcox et al., 1988). The present study showed that PDGF-A was identified as one of the transforming genes present in human plaque DNA and the expression of this gene can be induced by LDL (oxidized). HDL, a protective lipoprotein to atherosclerosis, did not modulate PDGF-A transcript levels in smooth muscle cells. While both oxidized LDL as well as PDGF-A transcripts are detected in atherosclerotic lesions this molecular mechanism could play a role in atherogenesis.

Furthermore, the present study provides strong evidence that disturbance of intercellular communication is another mechanism, which is probably important in the etiology of atherosclerosis. It is shown that compounds with atherogenic potential inhibit communication between human smooth muscle cells. The potency to modulate cell-cell communication correlates well with their atherogenic potential. For example, low density lipoproteins (LDL) in oxidized form and cholesterol oxidation products inhibited cell-cell communication. A non-atherogenic lipoprotein as high density lipoprotein (HDL) did not influence gap junctional communication between human smooth muscle cells. Otherwise, growth factors like PDGF and EGF can modulate cell-to-cell communication (Maldonado et al., 1988; Madhukar et al., 1989). This would imply that both paracrine and autocrine growth factor production are involved in atherogenesis, in which their influence on the gap junctional mediated growth control mechanism is a vital step in atherogenesis. At this stage, disruption of intercellular communication results in an uninhibited multiplication of these cells leading to the formation of (monoclonal) atherosclerotic lesions.

The main conclusion of the present study is that the three phenomena

1) production of growth modulating factors by vascular cells, including endothelium, monocytes, platelets and smooth muscle cells
2) DNA modification (e.g. PDGF-A) of smooth muscle cells
3) loss of intercellular communication

together are important factors in the process of atherosclerosis. The two main theories on atherosclerosis, the "monoclonal" and the "response to injury" theory, are compatible in many respects and can be fit into one unifying hypothesis as described in Fig. 8.1

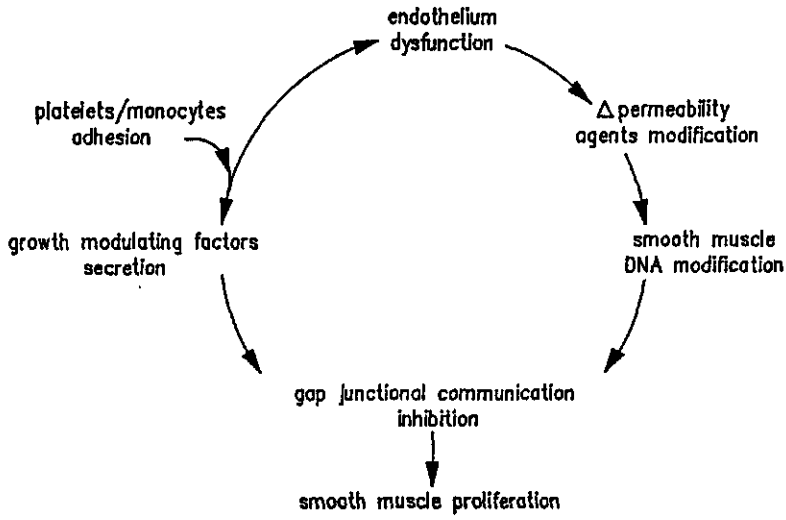

Fig. 8.1 Hypothetical scheme of main mechanisms involved in atherogenesis.

The figure shows that one of the earliest events in atherosclerosis is an increased adhesion of monocytes to what appears to be intact arterial endothelium, a phenomenon which can be well demonstrated using scanning electron microscopy (Faggiotto et al., 1984). In this process lipid factors like hypercholesteraemia and other factors as IL-1 or TNF (which introduces adhesion molecules on endothelial cells) are important triggers. As a consequence of monocyte emigration, there is focally increased permeability to LDL and macromolecules (Territo et al., 1984; Gerrity et al., 1979). An altered endothelial function (dysfunction) causing increased permeability may also be induced by risk factors such as hypertension, hyperlipidaemia, smoking, immunological factors, stress and diabetes mellitus (Reidy, 1985; Sieffert et al., 1981; Gordon et al., 1981, Munro and Cotran, 1988). In this way the agents present in the bloodstream (such as LDL) accumulate and are modified in the arterial wall and thereafter exert their effects locally

on vascular cells.

The next important step could be DNA modification(s) of smooth muscle cells as shown by the presence of transforming genes, as PDGF-A, in lesion DNA and the effects of the atherogenic LDL on cell transformation. This defect of an autocrine growth regulation is followed by another vital growth control mechanism, the gap junctional communication. Communication between human smooth muscle cells has been modified by several atherogens, like LDL (oxidized) and oxysterols. In case of impairement of both growth regulation mechanisms clonal growth may occur.

The processes described on the left side of the scheme are consistent with the paracrine proliferation mechanism of "the response to injury" theory of Ross (1990). The first trigger in this process is a dysfunction of the endothelium. This process introduces the adhesion and aggregation of platelets and/or monocytes to the endothelium, which results in a release of growth modulating factors as platelet-derived growth factor. At this stage, paracrine growth stimulating factors do not yet cause abnormal smooth muscle growth, because autonomous growth of cells still is controlled by gap junctional communication with surrounding cells. Only when growth factors (and atherogens) inhibit gap junctional communication abnormal smooth muscle cell proliferation occurs. Thus, inhibition of gap junctional intercellular communication could play a predominant role in the onset of atherogenesis.

A better understanding of these growth modulating processess will improve the possibility to study the possible atherogenic properties of chemicals within the framework of toxicological research.

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CHAPTER 9

SAMENVATTING EN SLOTBESCHOUWINGEN

Atherosclerose wordt gekenmerkt door verdikkingen in de arteriële vaatwand, genaamd plaques. De lesies bestaan uit gladde spiercellen, bindweefsel en veel extracellulaire lipiden. Gladde spiercelhyperplasie is een integrale gebeurtenis in de plaquevorming en wordt voornamelijk veroorzaakt door verstoringen van de autocrine en/of paracrine groei regulatie. In deze studie worden verstoringen van de intercellulaire communicatie via zogenaamde 'gap junctions' als een nieuw mechanisme, relevant voor de atherogenese, geïntroduceerd. De effecten van atherogenen op de communicatie tussen humane gladde spiercellen worden beschreven in de hoofdstukken 2, 3 en 4. De betrokkenheid van autocrine groeistimulatie in de atherogenese wordt duidelijk geïllustreerd door de bevinding dat geactiveerde transformerende genen aanwezig zijn in atherosclerotische lesies (hoofdstuk 5) en door de celtransformerende eigenschappen van het atherogene LDL (low density lipoproteinen), die beschreven staan in hoofdstuk 6. Hoofdstuk 5 toont tevens aan dat er verschillende transformerende genen in plaque-DNA aanwezig kunnen zijn, waarvan er één geïdentificeerd is als het PDGF-A (platelet-derived growth factor chain A) gen. De expressie van dit gen kan gemoduleerd worden door geoxideerd LDL (hoofdstuk 7).

Het onderhavige onderzoek verschaft extra argumenten voor de hypothese, dat autocrine productie van groeimodulerende factoren betrokken is bij de abnormale gladde spiercel-groei, die kenmerkend is voor de atherogenese. Er is onder andere aangetoond dat het atherogene LDL, in tegenstelling tot HDL, in staat is om fibroblasten *in vitro* te transformeren. Een directe aanwijzing voor genetische veranderingen van intimale cellen in de lesie werd gevonden door gebruik te maken van een DNA transfectietechniek. Er kon aangetoond worden dat plaque-DNA uit de humane aorta verschillende geactiveerde transformerende genen bevatte, waaronder PDGF-A. Dit is in overeenstemming met studies van Penn e.a. (1986) en Ahmed e.a. (1990), die transformerende genen in plaque-DNA uit humane kransslagaderen aantroffen. Zelfs DNA afkomstig uit gecultiveerde humane plaquecellen bleek celtransformerende capaciteit te bezitten (Parkes e.a., 1991). Alleen Yew e.a. (1989) kon geen transformerende potentie van plaque-DNA aantonen. De reden dat er tot nu toe tegenstrijdige resultaten verkregen zijn kan betekenen, dat er verschillende mechanismen aan de etiologie van atherosclerose ten grondslag kunnen liggen. In dit verband moet er nadrukkelijk op gewezen worden dat bij al deze studies

gebruik is gemaakt van gepoold plaquemateriaal en het dus niet noodzakelijk is dat er transformerende genen in élke plaque aanwezig zijn. De waargenomen transformerende capaciteit van plaque-DNA steunt de monoclonale hypothese van Benditt en Benditt (1973). Zij namen waar dat cellen afkomstig uit atherosclerotische lesies, een monoclonaal karakter hadden en verbonden daaraan de conclusie dat dit weefsel in het algemeen vergelijkbaar was met goedaardige tumoren. Als tegenargument wordt wel aangevoerd dat deze ogenschijnlijke monoclonaliteit veroorzaakt kan worden door sterke selectieve druk welke ten goede komt aan een subpopulatie van cellen, alhoewel bevindingen zoals de in plaque-DNA aanwezige transformerende genen (waaronder PDGF-A) en de transformerende eigenschappen van atherogenen dit tegenspreken. Additionele argumenten ten gunste van de monoclonale theorie zijn dat tumor-initiatoren --zoals chemische mutagenen en promutagenen (Albert e.a., 1975; Bond e.a., 1981), straling (Gold, 1961) en oncogene virussen (Minck e.a., 1979)- lesies in proefdieren kunnen induceren, die grote overeenkomsten vertonen met humane atherosclerotische plaques. Daarom kunnen de moleculaire veranderingen die ten grondslag liggen aan de proliferatie van gladde spiercellen in de atherogenese gelijkenissen vertonen met de moleculaire mechanismen, welke betrokken zijn bij de carcinogenese.

De gangbare opvatting over atherosclerose baseert zich op de 'response-to-injury' hypothese. Deze komt er in essentie op neer dat een paracrine stimulatie van gladde spiercelgroei het gevolg is van een beschadiging van het endotheel (Ross, e.a., 1976; Ross, 1981). Veel studies zijn in het raamwerk van dit hypothetisch model uitgevoerd. Steun voor deze hypothese is gebaseerd op de volgende waarnemingen. Bij proefdieren kunnen atherosclerotische lesies geïnduceerd worden door de endotheelcellaag te verwijderen. Beschadiging van het endotheel (veroorzaakt o.a. door mechanische, chemische, toxische, virale of immunologische verbindingen) veroorzaakte adhesie en aggregatie van bloedplaatjes gevolgd door afgifte van groeifactoren (zoals PDGF), welke aanzette tot migratie van gladde spiercellen naar de intima en proliferatie alsmede secretie van bindweefselcomponenten. In de loop der jaren is de hypothese gewijzigd (Ross, 1990). Ten eerste kwam aan het licht dat een verdwijning van de endotheelcellaag niet altijd optrad in de beginfase van de atherogenese (Davies e.a., 1976). Ten tweede is adhesie van bloedplaatjes niet noodzakelijk en zeker niet voldoende voor vorming van atherosclerotische lesies (Schwartz en Reidy, 1987). Tegenwoordig is de theorie als volgt:

a) het endotheel kan op verschillende stimuli reageren door subtiele functionele veranderingen (endotheliale dysfunctie) en/of door inductie van nieuwe endotheliale eigenschappen; een niet-cytotoxische beschadiging kan dus belangrijk zijn bij het ontstaan van atherogenese en b) bloedplaatjes zijn niet de enige initiatoren van gladde spiercel-proliferatie, aangezien groeibevorderende en groeiremmende factoren door andere celtypes (zoals macrofagen en endotheelcellen) uitgescheiden kunnen worden en zodoende effect kunnen hebben op de gladde spiercelgroei.

De 'response-to-injury' theorie kan echter niet alle waarnemingen verklaren, waaronder de bevindingen dat (intimale) gladde spiercellen zelf groeimodulerende factoren kunnen uitscheiden en hierop kunnen reageren en de celtransformerende eigenschappen van plaque-DNA. Dit duidt dus op de betrokkenheid van autocrine groeistimuli in de atherogenese. Een goede kandidaat voor autocrine groeifactorstimulatie van gladde spiercellen is PDGF-A. Transcriptieniveaus van dit transformerende gen zijn gelocaliseerd in intimale cellen van humane atherosclerotische lesies (Libby e.a., 1988; Wilcox e.a., 1988). Uit het onderhavige onderzoek blijkt dat het PDGF-A gen als één van de verschillende transformerende genen in humaan plaque-DNA aanwezig kan zijn en de expressie van dit gen kan door LDL (geoxideerd), in tegenstelling tot HDL, geïnduceerd worden. Omdat zowel geoxideerd LDL als PDGF-A transcripten in humane atherosclerotische lesies gedetecteerd zijn, kan dit moleculaire mechanisme een rol spelen in de atherogenese.

Een mechanisme dat niet eerder in verband is gebracht met de atherogenese is remming van de intercellulaire communicatie via zogenaamde 'gap junctions'. Het onderhavige onderzoek toont aan, dat een dergelijke remming een belangrijke rol kan spelen bij het ontstaan van atherosclerose. Er is gevonden dat verbindingen met atherogene potentie de communicatie tussen humane gladde spiercellen remmen. Ook is er een correlatie gevonden tussen de mate van modulatie van de cel-cel communicatie en de atherogene potentie van de verbindingen. Zo remmen bijvoorbeeld LDL (in geoxideerde vorm) en oxysterolen de communicatie via 'gap junctions' tussen human gladde spiercellen, terwijl HDL geen invloed hierop heeft. Anderzijds kunnen groeifactoren, zoals PDGF en EGF, ook de intercellulaire communicatie beïnvloeden (Maldonado e.a., 1988; Madhukar e.a., 1989). Dit leidt tot de conclusie dat zowel paracrine als autocrine groeifactorproductie betrokken kan zijn bij de atherogenese via de invloed van deze verbindingen op

communicatie met omringende cellen. Verstoring van de intercellulaire communicatie zou derhalve een kritische stap in de atherogenese zijn, die resulteert in een ongeremde celvermeerdering met als uiteindelijk resultaat de atherosclerotische lesie.

Samenvattend leidt het in dit proefschrift beschreven onderzoek tot de conclusie dat in essentie modulering van de volgende drie processen bepalend is voor het ontstaan van atherosclerose:

1) produktie van groeimodulerende factoren door vaatwandcellen, waaronder endotheelcellen, monocyten, bloedplaatjes en gladde spiercellen
2) DNA modificatie (o.a. PDGF-A) van gladde spiercellen
3) intercellulaire communicatie

De twee voornaamste theorieën omtrent atherosclerose, de 'monoclonale' en de 'response-to-injury' theorie, zijn in vele aspecten verenigbaar en kunnen geïntegreerd worden in een hypothetisch model zoals schematisch beschreven in Fig. 9.1.

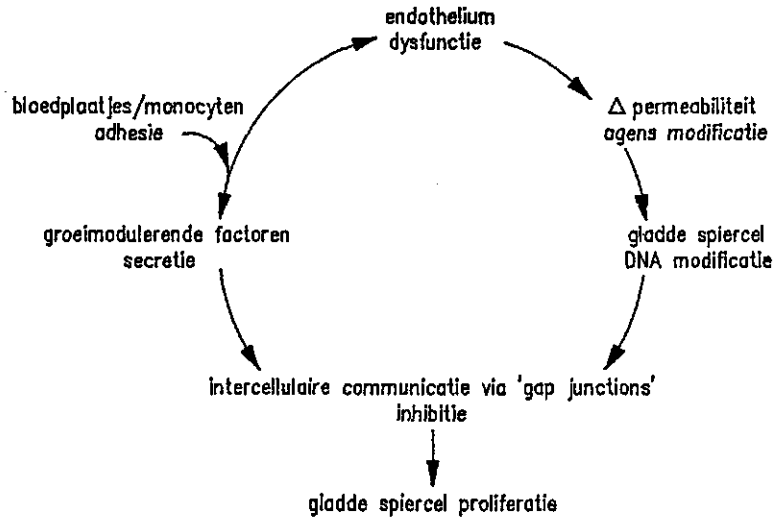

Fig 9.1 Hypothetisch schema van de belangrijkste mechanismen betrokken bij de atherogenese

De figuur toont aan dat een verhoogde adhesie van monocyten (aangetoond met scanning electronenmicroscopie) een van de eerste gebeurtenissen in de atherogenese is (Faggiotto e.a., 1984). Belangrijke triggers in dit proces zijn naast lipiden (bijvoorbeeld hypercholesteraemie) andere factoren, zoals IL-1 en TNF die adhesiemoleculen op de endotheelcellen introduceren. Als gevolg van emigratie van monocyten wordt de permeabiliteit van het endotheel voor LDL en macromoleculen vergroot (Territo e.a., 1984; Gerrity e.a., 1979). Een veranderde functie van het endotheel (dysfunctie) gepaard gaande met een verhoogde permeabiliteit kan ook veroorzaakt worden door verschillende risicofactoren, waaronder hypertensie, hyperlipidaemie, roken, immunologische factoren, stress en diabetes mellitus (Reidy, 1985; Sieffert e.a., 1981; Gordon e.a., 1981; Munro en Cotran, 1988). Op deze manier accumuleren agentia (o.a. LDL) uit het bloed in de vaatwand, waar deze vervolgens gemodificeerd kunnen worden en hun effect lokaal op vaatwandcellen kunnen uitoefenen.

De volgende belangrijke stap zou DNA modificatie van gladde spiercellen kunnen zijn. Belangrijke argumenten hiervoor zijn, dat transformerende genen (o.a. PDGF-A) in plaque-DNA aanwezig zijn en dat het atherogene LDL celtransformerende eigenschappen bezit. Deze verstoring van een autocrine groeiregulatie kan gevolgd worden door een inhibitie van communicatie via 'gap junctions'. Gevonden is dat communicatie tussen humane gladde spiercellen gemodificeerd kan worden door verschillende atherogenen, waaronder LDL (geoxideerd) en oxysterolen. Het model impliceert dat in geval van verzwakking van beide groeiregulatiemechanismen clonale groei kan optreden.

De processen beschreven aan de linkerzijde van het schema zijn consistent met het paracrine proliferatiemechanisme, dat kenmerkend is voor de 'response to injury' theorie van Ross (1990). De eerste trigger in dit proces is een dysfunctie van het endotheel. Dit leidt vervolgens tot adhesie en aggregatie van bloedplaatjes en/of monocyten aan het endotheel met als gevolg een afgifte van groeimodulerende factoren, zoals PDGF. In deze fase veroorzaken paracrine groeifactoren nog geen ongecontroleerde gladde spiercelgroei, omdat autonome celgroei nog steeds wordt voorkomen door de groeiregulerende invloed van de omringende cellen. Echter indien groeifactoren (en atherogenen) de communicatie met omringende cellen via 'gap junctions' remmen kan abnormale gladde spiercelgroei optreden.

Toegenomen inzicht in de groeimodulerende processen zal betere mogelijkheden bieden eigenschappen van stoffen te onderzoeken die relevant zijn voor de atherogenese in het kader van toxicologisch onderzoek.

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CURRICULUM VITAE

Renate Zwijsen werd geboren op 24 mei 1962 te Geldrop en behaalde in 1980 het diploma Atheneum-B aan het St. Joriscollege te Eindhoven. In datzelfde jaar werd met de studie Human Voeding aan de Landbouwuniversiteit te Wageningen begonnen. Tijdens deze studie deed zij de hoofdvakken Toxicologie en Gezondheidsleer en een bijvak Humane Voeding. Haar stageperiode heeft ze doorgebracht bij de afdeling Biologische Toxicologie van het Instituut voor Toxicologie en Voeding (ITV) te Zeist. In 1987 werd de universitaire studie met lof afgerond.

Sedert 1 januari 1988 is zij aan dit promotieonderzoek werkzaam bij de vakgroep Toxicologie van de Landbouwuniversiteit te Wageningen. De resultaten staan beschreven in dit proefschrift.