Modulation of Gap Junctional Intercellular Communication between Human Smooth Muscle Cells by Leukocyte-derived Growth Factors and Cytokines in relation to Atherogenesis

Promotor: Dr J.H. Koeman
Hoogleraar in de Toxicologie

Modulation of Gap Junctional Intercellular Communication between Human Smooth Muscle Cells by Leukocyte-derived Growth Factors and Cytokines in relation to Atherogenesis

Anne Mensink

Proefschrift

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Contents

Stellingen

1. De aanhoudende remming van de intercellulaire communicatie via gap junctions (GJIC) door cytokinen als TNFα en IFN-γ wijst erop dat deze faktoren een belangrijke rol kunnen spelen in de atherogenese.
Dit proefschrift

2. Macrofagen kunnen GJIC tussen nabijgelegen gladde spiercellen moduleren.
Dit proefschrift

3. De door TNFα geïnduceerde remming van GJIC tussen gladde spiercellen wordt gemedieerd door superoxide radicalen.
Dit proefschrift

4. Er bestaat geen simpel, rechtlijnig verband tussen de remming van GJIC en de stimulatie van celproliferatie door endogene faktoren.
Rudkin et al., 1996, J. Cell. Physiol. 168:433-441.
Madhukar et al., 1989, Carcinogenesis 10:13-20.
Dit proefschrift

5. Conclusies die verbonden worden aan het onderzoek van Parkes et al. (1991) en Marin et al. (1993) betreffende *myc* genexpressie in gezond en atherosclerotisch vaatweefsel zijn niet valide, daar de juiste controles ontbreken.
Parkes et al., 1991, Am. J. Pathol. 138:765-775.
Marin et al., 1993, J. Vasc. Surg. 18:170-177.

6. De termen 'Functional Foods' en 'Health Foods' zijn slecht gekozen, daar zij eventuele schadelijke effekten van de betreffende produkten op de gezondheid aan het oog onttrekken.

7. De term 'aderverkalking' dient vervangen te worden door de term 'slagadervervetting'.

8. De ontwikkeling van nieuwe antibiotica en vaccins verdient een hogere prioriteit dan tot nu toe aan dit onderzoek toegekend is.

9. De gevaarlijkste hartziekten zijn nog steeds: afgunst, haat en hebzucht.
Pearl S. Buck

10. Het opnemen van vrouwen in de Commissie-Verruijt van de Koninklijke Nederlandse Akademie van Wetenschappen had wellicht meer kunnen bijdragen aan een vrouwvriendelijker imago van de bèta-vakken dan de door deze Commissie gedane aanbevelingen hieromtrent.

11. Goede en slechte eigenschappen zijn ook op (sub)cellulair niveau vaak nauw met elkaar verbonden.

12. Geheel onverwacht bleek bier structuur aan dit proefschrift te kunnen geven.

Stellingen behorende bij het proefschrift:

Modulation of gap junctional intercellular communication between human smooth muscle cells by leukocyte-derived growth factors and cytokines in relation to atherogenesis

Anne Mensink
Wageningen, 26 november 1997

Chapter 1
General Introduction

Introduction

A variety of food components and environmental factors contribute to the development of the widespread arterial disease named atherosclerosis. The present line of research was established in order to explore mechanisms and processes in the vascular wall that might be vulnerable to potentially toxic contaminants to which the human population is exposed. Previous work broadened the knowledge on the parallelism of mechanisms in carcinogenesis and atherogenesis (1). Emphasis was layed on the role of gap junctional intercellular communication (GJIC) in the pathogenesis of atherosclerosis. In the present study, the involvement of GJIC in this disease process was further explored. Special attention was given to the effects of endogenous factors on GJIC between vascular cells and to interactions between different cell types of the artery wall. The knowledge obtained in this study may serve as a basis for subsequent studies on the role of toxic chemicals in atherogenesis and other inflammatory diseases.

Atherosclerosis

Atherosclerosis is an arterial disease with a slow progression. The narrowing of the lumen of these blood vessels gives rise to myocardial and cerebral infarctions, which are among the major causes of morbidity and mortality in Western societies. Multiple risk factors have been described for the development and acceleration of atherosclerosis. Genetic, environmental and life-style factors may contribute to the pathogenic process. Cigarette smoke, hypertension, hyperlipidaemia, obesity, diabetes mellitus and physical inactivity are for instance all well known factors with atherogenic potential.

Atherosclerotic lesions - their morphology

The arterial vessel wall is build up of three layers: intima, media and adventitia. Endothelial cells separate other elements of the innermost layer of the artery wall, the intima, from the blood. Smooth muscle cells (SMC), scattered macrophages and extracellular matrix components are part of the healthy intima as well (2). The media consists mainly of SMC and connective tissue. The outermost layer of the artery wall, the

adventitia, is composed of fibroblasts and connective tissue and contains small blood vessels, lymph vessels and nerves. In the intima, the process of atherogenesis originates.

Atherosclerosis is characterized by the occurrence of focal thickenings of the intimal layer in the artery wall. In the course of the disease process, blood monocytes and T lymphocytes infiltrate into the intima (3-6) after which monocytes differentiate into macrophages. A substantial number of intimal macrophages take up lipid droplets. The uptake of cholesterol(esters) causes these macrophages and some SMC to transform into foam cells (7). Lesion progression is also accompanied by the indolent proliferation of SMC and macrophages (8-12), changes in extracellular matrix synthesis and accumulation of intra- and extracellular lipids (7,13). As a result of these processes, atherosclerotic plaques increase in size and may impede the flow of blood and occlude the arteries concerned.

Atherosclerotic lesions can be classified into several morphologically different types (7,14). Early lesions such as fatty streaks (aggregations of macrophage-derived foam cells and T lymphocytes) and more advanced lesions like fibrous plaques (a dense cap of connective tissue with embedded SMC, macrophages, foam cells and T lymphocytes overlays a core of lipid, necrotic debris and calcium) have been described. It is thought that early lesions proceed to more advanced lesions in the course of time, possibly under the influence of an additional stimulus (7,15).

Pathophysiological aspects of atherosclerosis

Atherogenesis is a pathological process with great complexity. As a result, a number of hypotheses concerning its etiology arose. Lipids, especially low density lipoprotein (LDL) (16,17), blue-green algae (18), hypoxia (19) and viruses (20-23) have been suggested to be possible etiologic factors in the development of atherosclerosis. Furthermore, immune and inflammatory mechanisms seem to be potent modulators of the atherosclerotic process (24-26). These hypotheses however, are not by definition mutually exclusive, since they may reflect different aspects of lesion formation (16,17). There are two current theories in which aspects of the above-mentioned hypotheses fit.

The most accepted theory is the response-to-injury hypothesis (27-29). This theory is supported by animal studies, by *in vitro* studies of arterial cells and by observations in human atherosclerotic plaque material obtained at surgery. Central to the response-to-injury hypothesis is the proposal that mechanical, chemical, toxic, viral or immunological agents may cause endothelial cell dysfunction, resulting in an increased migration of monocytes and T lymphocytes into the intima. Lipid accumulation results in the formation of macrophage foam cells and a fatty streak is formed. As a consequence of cellular

interactions between the cell types present in atherosclerotic lesions the fatty streak transforms into a fibrous plaque. Growth factors and cytokines produced by endothelial cells, SMC, macrophages, foam cells and T lymphocytes may act in an autocrine and/or paracrine manner on SMC. This may result in an abnormal SMC proliferation and accumulation in the intima and may affect lipid and protein synthesis, including the formation of extracellular matrix components.

The other (less supported) theory is the monoclonal hypothesis (30-34). According to this theory, each atherosclerotic lesion develops out of one single mutated SMC, analogous to the process of tumorigenesis. As a consequence, plaque formation is divided in stages of initiation, promotion and progression. In the initiation phase, exogenous chemicals, ionizing radiation, viral agents or perhaps even endogenous substances may induce mutations in the SMC DNA. During the following promotion phase, (clonal) SMC proliferation occurs. Chemical, nutritional, hormonal or even mechanical factors may influence this SMC proliferation. Experiments in which tumor initiators like chemical (pro)mutagens (35-37), radiation (37,38) and oncogenic viruses (39-42) were able to induce or accelerate atherosclerotic lesion formation in laboratory animals support the monoclonal hypothesis. Plaques developed in cockerels when they were treated with 7,12-dimethylbenzo[a]anthracene and methoxane in an 'initiation-promotion sequence' experiment (43). Furthermore, epidemiological literature indicated that certain carcinogenic agents may exert an atherogenic effect in humans as well (44). Experiments demonstrating that human plaque DNA was able to transform mouse fibroblasts (45-47) provided additional evidence for the monoclonal hypothesis. Experiments with human plaque SMC revealed that the transforming gene(s) resided in the SMC and that they were retained during passage in culture (48). However, other researchers failed to observe the transforming activity of human plaque DNA (49).

Both the response-to-injury theory and the monoclonal hypothesis indicate that disturbance of SMC growth control mechanisms may be seen as a key event in the pathogenic process. Therefore, mechanisms and factors involved in growth control deserve some additional notice. Two general systems regulating cell growth, namely gap junctional intercellular communication (GJIC) and the network of growth factors and cytokines, will be discussed.

Involvement of gap junctional intercellular communication

Gap junctions are transmembrane channels that span plasma membranes of two adjacent cells (50-52). They often aggregate into clusters. Gap junctions allow transport of ions and other small water-soluble molecules ($\leq$ 1000 D) of one cell to another. This

passage of factors includes amino acids, sugars and signal molecules like cyclic nucleotides, Ca^{2+} and inositol triphosphate. Gap junctions are formed by head-to-head alignment of two hemi-channels (connexons) on opposing cells. Connexons consist of six subunit proteins named connexins. Several different gap junction connexin proteins exist (53). Each connexin gene has its own distinct pattern of tissue-specific expression. However, multiple connexins may be expressed in the same cell type. Gap junctions may be formed between cells expressing the same connexin proteins, but also between cells expressing different types of connexins.

In vivo studies demonstrated that vascular SMC are interconnected by gap junctions (54-56). Immunohistochemistry revealed connexin 40 (Cx40) and connexin 43 (Cx43) protein expression in relatively small clusters in the plasma membranes. *In vitro* studies confirmed that GJIC occurred between human and rat SMC (57-63). Electron microscopy, Northern blot analysis and/or immunohistochemistry demonstrated the presence of Cx43 in cultured SMC from human or animal origins (63-67). Cx40 RNA could also be detected in SMC (65). Unlike Cx43 expression however, the Cx40 expression appeared to diminish strongly with successive passages of primary SMC in culture. In addition it was demonstrated that the level of Cx43 expression of SMC in culture is not a fixed characteristic too. Only a few gap junctions were found in SMC of the contractile phenotype, a state in which cell proliferation does not occur. Cells that had been modulated to a synthetic phenotype, a state in which SMC may proliferate, expressed numerous gap junctions at cell-cell interfaces (67). Cx46, Cx45, Cx42, Cx32 and Cx26 mRNA could not be detected in hybridization and/or immunohistochemistry experiments with SMC (63,66).

The extend of GJIC may be regulated by two major mechanisms. Firstly, through the synthesis, degradation and incorporation of connexins into the plasma membrane, and secondly, via local regulation of the opening and closing of gap junction channels. Intracellular messengers like cAMP, Ca^{2+} and H^{+} ions influence gap junction permeability in direct and/or indirect ways. Indirect effects of these molecules on channel permeability include pathways involving calmodulin dependent protein kinase, protein kinase A (PKA) and protein kinase C (PKC), which may phosphorylate connexin proteins. Phosphorylation may affect gap junction permeability by a shift in the tertiary structure of the connexin (68).

GJIC is regarded as an important mechanism in the control of cell growth, cell differentiation and tissue homeostasis (69,70). Therefore, transient or permanent modulations of GJIC may have pathophysiological consequences. GJIC has long been postulated to be involved in carcinogenesis. *In vitro* and *in vivo* studies (71-78) provide ample evidence that inhibition of GJIC and changes in the regulation of GJIC play a role in

tumor promotion. A broad range of experimental approaches provided evidence that: (a) most tumor cells show an aberrant GJIC among themselves and/or with surrounding normal cells, (b) most tumor promoting chemicals (reversibly) inhibit GJIC, (c) several anti-tumor promoting agents upregulate GJIC, (d) tumor promoter-mediated inhibition of GJIC is associated with enhancement of cell transformation, (e) connexin genes suppress malignant cell growth after transfection, (f) oncogenes are correlated with the downregulation of GJIC, (g) tumor suppressor genes are correlated with upregulation of GJIC or with the prevention of downregulation of GJIC.

There are indications that altered GJIC may be important in atherogenesis as well. In human atherosclerotic lesions, Cx43 expression was observed. In one study (79), early atherosclerotic lesions showed markedly increased expression of Cx43 gap junctions between intimal SMC as compared with undiseased vessels. As the disease progressed to more advanced plaques, however, the quantity of junctions declined, ultimately to levels below those of the undiseased vessel wall. In this study, Cx43 protein expression could not be contributed to macrophages. Another study (80) however, revealed that macrophage-foam cells in human atherosclerotic arteries displayed a strong Cx43 mRNA expression. In experiments with hypercholesterolemic rabbits, Cx43 mRNA and protein expression was upregulated in macrophage-foam cells, whereas Cx43 mRNA expression in SMC declined when compared to normal control animals (81). Furthermore, atherogens like oxidized LDL, cigarette smoke condensate, oxysterols and aldehydes were able to reduce GJIC between SMC (57-60) suggesting that disturbance of gap junctional-mediated growth control by these atherogens might contribute to the SMC proliferation seen in atherosclerotic lesions.

In addition to the up- or downregulation by exogenous chemicals, modulation of GJIC occurs under the influence of endogenous factors, such as hormones and growth factors as well (70).

Involvement of growth factors and cytokines

Growth factors and cytokines are implicated in a wide range of physiological and pathological processes, which include growth and development, immune responses, tissue repair, atherosclerosis and neoplasia (82). Growth factors and cytokines are hormone-like polypeptides, which are produced by a variety of different cell types. They generally act locally or over relatively short distances. Their biological actions are pleiotrophic and redundant; each factor exerts multiple effects on different cells, and different factors can act on the same cell to induce similar effects (83,84).

Growth factors and cytokines exert their effects by binding to specific receptors on

the cell surface of target cells. This binding induces signal transduction mechanisms that evoke specific responses dependent on the responding cell type, the stimulating factor and the cellular environment. Cell - matrix interactions and the local concentration of growth factor or cytokine binding proteins may regulate the ability of a cell to respond to a given factor as well (85).

The release of growth factors and cytokines may stimulate neighbouring cells in a paracrine or juxtacrine way or may influence the cell type itself in an autocrine fashion when the cell type producing that factor also expresses the corresponding receptor. Several factors may act simultaneously on cells; the net effect of these factors may depend on the balance between induced mechanisms. The timing of growth factor and/or cytokine release, the responsiveness of target cells and the location of producing and responding cells may all be of importance in the network of positive and negative regulatory signals.

The possible roles of cytokines and growth factors in the development of atherosclerosis is comprehensively discussed in several reviews (85-87). A short summary is given here. A substantial number of different growth factors and cytokines have been identified in atherosclerotic lesions. In normal arteries, many of these growth factors and cytokines are expressed in low or undetectable levels. In plaques however, increased levels of most factors were observed. This may be the result of the infiltration, transformation and activation of leukocytes in the atherosclerotic intima. However, cells normally present in the artery wall (endothelial cells and SMC) also contribute to the expression of growth factors and cytokines in the process of atherogenesis.

Several factors are capable of inducing SMC migration and/or proliferation and thus of participating in the pathogenesis of atherosclerosis. Other effects of growth factors and cytokines, such as alteration of lipid metabolism and connective tissue synthesis, may contribute to atherosclerotic lesion formation as well. Furthermore, the effects of growth factors and cytokines on monocyte-chemoattraction, macrophage activation and cell survival seem to play a role in the pathogenic process too.

In the study described in this thesis, tumor necrosis factor α (TNFα), interferon-γ (IFN-γ), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and interleukin-6 (IL-6) were chosen as representatives of several classes of growth modulating factors. Their presence in atherosclerotic lesions and effects on SMC are now expounded in some detail.

TNFα

Biologically active human TNFα is a non-glycosylated protein consisting of two or three identical subunits with molecular masses of 17 kD (88-91). Although originally characterized as a factor with antitumor activity, TNFα is now seen as a factor involved in several physiological and pathological conditions, like inflammation, immuno-pathological processes, tissue injury and wound healing. Monocytes and macrophages are important cellular sources of TNFα, although other cell types can produce TNFα as well. This cytokine acts on many cell types by binding to specific high-affinity receptors. Two TNFα receptors with molecular masses of 55 kD and 75 kD have been identified. After binding of TNFα to its receptors, the complex is rapidly internalized and degraded and signal transduction pathways are set in motion. These post-receptor mechanisms include G protein activation, protein phosphorylation, phospholipase activation and oxygen radical production. Some TNFα-induced signalling mechanisms and related biochemical effects seem to be cell type-dependent.

Immunohistochemical analysis showed that TNFα is present in human atherosclerotic lesions in elevated amounts as compared with the normal vessel wall (92-95), both in cells and as extracellular deposits in the connective tissue matrix. TNFα positive staining was found in the cytoplasm of macrophages and mast cells, but also in the cytoplasm of intimal SMC which suggests that these cell types are capable of producing TNFα. TNFα positive staining was also found attached to the cell membrane of SMC, indicating that SMC may be a target for the cytokine as well. The distribution pattern of TNFα mRNA varied within different parts of the plaque micro-environment (96). When macrophages from carotid plaques were isolated and were cultured *in vitro*, they spontaneously produced substantially more TNFα than corresponding blood monocytes (97). Stimulated SMC in culture also synthesize and secrete biologically active TNFα (98,99). Acetylated LDL and oxidized LDL stimulate TNFα expression and release by human monocyte-macrophages *in vitro* (100,101).

In vitro studies revealed that SMC characteristics may be influenced by TNFα. In table 1.1 some effects with relevance for the process of atherosclerosis are given.

Table 1.1.: Effects of TNFα on cultured SMC

effect	SMC species	references
proliferation:		
stimulation	h,rb	102-106
no effect	h	107
inhibition	m,b,r	108-110
induction of:		
IL-1 production	h	103
IL-6 production	h	111
GM-CSF and M-CSF production	h	112
mRNA 2'-5' oligoadenylate synthetase	h	98
prostaglandin E2 release	h	98
matrix metalloproteinases	h	113
adhesion molecule expression	h	114-116
migration	h	117
apoptosis (together with IFN-γ)	h,r	118
NO production (together with IFN-γ)	r	119
NOS mRNA expression	r	120,121
alterations of glycosaminoglycans	b	122
scavenger receptor expression	rb	123
modulation of phenotype	rb	106

h = human; r = rat; rb = rabbit; b = bovine; m = mouse

IFN-γ

Biologically active human IFN-γ (type II IFN) is a homodimer of two polypeptides with molecular masses of 17 kD (124). Although IFN-γ first was recognized on the basis of its antiviral activity, this cytokine is now seen as a factor involved in many immune and inflammatory responses. IFN-γ is secreted mainly by activated T lymphocytes and is regarded as an important macrophage activating factor. This cytokine exerts its effect on cells through binding to a specific receptor expressed at cell surfaces. There appears to be only one single type of IFN-γ receptor that is expressed on nearly all cell types of the body. IFN-γ induces dimerization of its receptor; after binding the ligand-receptor complex is internalized and signal transduction pathways are set in motion. Post-receptor

mechanisms involving protein kinases, receptor phosphorylation and ion fluxes have been described. However, a signal transduction mechanism proposing that intracellular IFN-γ itself induces cellular responses has been described as well.

Immunohistochemical analysis showed that IFN-γ is present in human atherosclerotic lesions in and around some T lymphocytes (4). Oxidized LDL stimulated peripheral blood mononuclear cells *in vitro* to produce IFN-γ (125). Cell culture studies revealed that SMC characteristics may be influenced by IFN-γ. In table 1.2 some effects with relevance for the process of atherosclerosis are given.

Table 1.2.: Effects of IFN-γ on cultured SMC

effect	SMC species	references
proliferation:		
stimulation	h	126
inhibition	h,r,m	102-104,108, 110,127-130
induction of:		
M-CSF expression	h	112
adhesion molecule expression	h,rb	115,131
HLA / MHC expression	h,r	127,132,133
mRNA 2'-5' oligoadenylate synthetase	h	129
apoptosis (together with TNFα)	h,r	118
PDGF-ß receptors	h	126
interferon-inducible protein-10	r	134
NOS mRNA expression	r	120,121
NO production (together with TNFα)	r	119
phenotypic modulation	r	135
scavenger receptor expression	rb	123
inhibition of:		
phenotypic transformation	h	136
α-SM actin production	r	128

h = human; r = rat; rb = rabbit; m = mouse

IL-6

Human IL-6 is a glycoprotein with a molecular mass of ~26 kD (137-140). IL-6 has a wide variety of biological activities and is involved in inflammation and some lymphoid malignancies, induces differentiation and/or gene expression and regulates cell growth in a cell type-dependent manner. IL-6 is produced by monocytes and macrophages, but also by many other cell types. This cytokine exerts its effect by binding to a specific cell surface receptor capable of expressing both high and low affinity binding sites. After binding of IL-6 to the ligand binding chain, association with a nonligand binding chain responsible for signal transduction occurs. Therefore, the IL-6 receptor consists of two polypeptide chains. Little is known about the intracytoplasmatic signalling mechanisms; however, tyrosine kinase activity seems to play an important role.

Reverse transcription polymerase chain reaction and *in situ* hybridization techniques showed that IL-6 gene transcripts are expressed in human atherosclerotic lesions. In one study, atherosclerotic arteries expressed extensively higher levels of IL-6 mRNA as compared with non-atherosclerotic arteries (141). Another study revealed that lesions did not per definition contain elevated levels of IL-6 mRNA (142). Immunohistochemical staining indicated that IL-6 is present in human atherosclerotic walls as cellular and extracellular deposits in the connective tissue matrix (143). Normal intima presented only cellular deposits in contrast with plaques where extended extracellular deposits were found as well, suggesting that an increased synthesis and release takes place in atherosclerotic lesions. Double immunostaining methods revealed that in fatty streaks and more advanced lesions, both macrophages and SMC expressed IL-6 (144). Cultured SMC are able to synthesize and secrete IL-6 as well (111,145). Several *in vitro* studies demonstrated that SMC may be influenced by IL-6 (table 1.3).

Table 1.3.: Effects of IL-6 on SMC *in vitro*

effect	SMC species	references
proliferation:		
stimulation	r	146-148
no effect	h	111
induction of:		
PDGF production	r	148
c-myc mRNA level	r	146

h = human; r = rat

PDGF

PDGF, a glycoprotein of approximately 30 kD, was first isolated from human platelets (149,150). This platelet-derived molecule consisted of two polypeptide chains, denoted A and B, held together by disulphide bonds. These A and B chains are 60% homologous with one another when their amino acid sequences are compared and have molecular weights of 16 kD and 14 kD respectively. Many other cell types can synthesize PDGF as well; however, they usually secrete PDGF as homodimers of the A or the B chain. PDGF is seen as a factor involved in normal growth, wound healing, inflammatory responses and malignancies.

PDGF is a mitogen for a broad spectrum of cells, including connective tissue forming mesenchymal cells. PDGF induces its biological effects on cells through binding to specific high-affinity receptors on cell surfaces. The PDGF receptors are homo- or heterodimers consisting of α and ß subunits, which are brought together by one of the three isoforms of PDGF (PDGF-AA; PDGF-BB; PDGF-AB). The α subunit can bind to either PDGF-A or PDGF-B chain; the ß subunit only binds to PDGF-B chains. After binding of PDGF to the appropriate combination of receptor units, post-receptor mechanisms are set in motion. Phosphatidylinositol breakdown, formation of eicosanoids, formation of membrane-associated diglycerides, activation of PKC and calcium mobilization have amongst others been reported to play a role in PDGF signal transduction.

Hybridization studies identified the presence of PDGF-A and PDGF-B gene transcripts in human atherosclerotic plaques at levels greater than the levels detected in normal arteries (142,151). In one study with cell type-specific markers, the predominant cell type found to express PDGF-B mRNA was the endothelial cell; little or no expression of PDGF-B mRNA was detected in macrophages (152). PDGF-A mRNA expression was attributed to mesenchymal-appearing intimal cells. Another study also demonstrated that the majority of PDGF-A transcripts were produced by SMC (153). However, this study suggested that the bulk of PDGF-B transcripts was produced by activated macrophages in the lesion. This suggestion was supported by immunohistochemical experiments which demonstrated that many non-foam cell macrophages in lesions contained PDGF-B protein (10,154). PDGF receptor mRNA expression was observed in intimal mesenchymal cells of human atherosclerotic lesions, but not in medial SMC (152). PDGF-ß receptors were found on intimal SMC in atherosclerotic lesions by immunohistochemical techniques. PDGF-ß receptors were not detected in SMC in the media or in regions of the intima unaffected by atherosclerosis (155). Macrophages and SMC are both capable of synthesizing PDGF *in vitro* (151,156-160). Both native and oxidized LDL enhance PDGF-A gene transcripts in cultured SMC (161,162). Decreased PDGF-B mRNA expression however, was observed when monocyte-macrophages were exposed to oxidized LDL. In contrast,

IFN-γ enhanced the level of PDGF-B mRNA transcripts in macrophages (163). Cell culture studies revealed that SMC characteristics may be influenced by PDGF (see table 1.4).

Table 1.4.: Effect of PDGF on cultured SMC

effect	SMC species	references
stimulation of proliferation	h h r rb	103,126,129, 164,165 166-171 172
induction of:		
IL-6 production	h	111
bFGF production	b	173
MAP kinase activation	h	174
collagenase production	h	175
H_2O_2	r	176
phospholipase D activation	r	166,177,178
inositol triphosphate release	r	167,179
diacylglycerol production	r	167,179
calcium mobilization	r	167,179
pH changes	r	167,179
activation of PKC	r	166
c-fos, c-myc genes	r	168
PLC phosphorylation	rb	172
phenotypic transformation	rb	180
migration	h,b	117,173
LDL binding	mo	181
inhibition of:		
adhesion molecule expression	h	115
apoptotic cell death	h	182
NO induction by cytokines	r	183,184

h = human; r = rat; rb = rabbit; b = bovine; mo = monkey

bFGF

bFGF is a polypeptide with a molecular weight of 16 - 18 kD (185). It belongs to the family of heparin-binding growth factors and is therefore often bound to heparin-like molecules in the extracellular matrix and basement membranes. bFGF is widely distributed in tissues where it is involved in mitogenesis, chemotaxis and differentiation; for example in embryonic development, angiogenesis and tumorigenesis. bFGF is synthesized by many cell types. It is a cellular, rather than a secreted protein; its association with cells, extracellular matrix and basement membranes suggests that bFGF is a 'stored' growth factor. In inflammation or injury, bFGF is released by heparanases and proteases, produced by macrophages, for instance. Cells that respond to bFGF have been shown to posses specific FGF receptors. Four members of the FGF receptor family have been identified. Both high and low affinity binding sites exist. Signal transduction pathways implicated in bFGF-induced gene activation include guanylate cyclase induction (186), generation of diacylglycerol and subsequent PKC activation (187-189), Ca^{2+} mobilization (187,190), increased tyrosine kinase activity and protein phosphorylation (190-193) and activation of the Na^{+}/H^{+} antiport (190). Different cell types appear to use different post-receptor transduction pathways, thus, a general mechanism cannot be given.

Immunohistochemical studies showed that bFGF is expressed in human atherosclerotic plaques (194). In contrast to the expression in normal arteries where bFGF was detected in the media and adventitia, the atherosclerotic intima also reacted for bFGF. bFGF positive staining was found in foam cells, macrophages and SMC. The relative amount of bFGF in plaques versus normal arteries remains controversial however, since other studies revealed that atherosclerotic arteries contained less immunoreactive bFGF protein than control vessels (195). Members of the FGF receptor family were localized in vascular SMC of both normal and diseased human arteries by means of immunolocalization (195,196). Receptor expression was also found in foam cells and macrophages. Cultured human and bovine SMC expressed and synthesized bFGF (197-199). Cholesteryl ester or 25-hydroxycholesterol enrichment enhanced bFGF synthesis and release in rabbit and bovine SMC (200). *In vitro* studies revealed that SMC characteristics may be influenced by bFGF. In table 1.5 some effects with relevance for the atherogenic process are given.

Table 1.5.: Effects of bFGF on cultured SMC

effect	SMC species	references
stimulation of proliferation	h,r,b	199,201-204
induction of:		
collagenase expression	h	205
mRNA LDL receptor	r	206
acyl-CoA cholesterol acyltransferase	r	206
cholesterol esterification	r	206
protein tyrosine phosphorylation	r	206
PKC and PKA activity	r	206
H_2O_2	r	176
NO production by cytokines	r	184
inhibition of:		
type I collagen gene expression	h	205

h = human; r = rat; b = bovine

Association of growth factors or cytokines and GJIC

Growth factors and cytokines bring about a form of intercellular communication by binding to specific receptors at cell surfaces. Apart from this receptor-mediated signalling mechanism, direct intercellular communication occurs via gap junctions in plasma membranes. In contrast to the wealth of data available on both forms of intercellular communication, little is known about the linkage of these two phenomena.

Several growth factors and cytokines have been reported to modulate GJIC when applied to cultured cells (table 1.6). Modulation of connexin expression or connexin phosphorylation, modulation of intracellular pH and activation of PKC have been proposed as mechanisms regulating some of these growth factor or cytokine-induced effects on GJIC.

To date, reports in literature have been fragmentary. A limited number of growth factors and cytokines have been examined for their effects on GJIC. Furthermore, it is difficult to draw a conclusion or to make a comparison between these factors, since divergent culture conditions like different concentrations and various exposure times have been applied. Moreover, unrelated cell types have been used frequently. Interactive effects on GJIC have only been reported concerning epidermal growth factor (EGF) and

transforming growth factor ß (TGFß) or hepatocyte growth factor (HGF) and $TGF\beta_1$. These interactive effects were shown to be cell type-dependent (210,211,219,220,222).

Table 1.6.: Effects of growth factors or cytokines on GJIC

growth factor or cytokine	cell type	effect on GJIC	references
bFGF	BME	↑	207
	rat cardiac fibroblasts	↑	208
	rat cardiac myocytes	↓	209
EGF	NRK	↑,↓	210,211
	Balb C 3T3	↓	211
	T51B	↓	212,213
	NHEK	↓	214
	K7	↑	215
IL-1α	HUVEC	↓	216
BMP-2	MC3T3-E1	↓	217
BMP-3	MC3T3-E1	↓	217
NDFß	rat Schwann cells	↑,↓	218
PDGF	NRK	↓	211
	Balb C 3T3	↓	211
PDGF-BB	C3H/10T1/2	↓	219
TGFß	NRK	↓	211
	Balb C 3T3	↑	211
	MC3T3-E1	↓	217
	BE	↓	220
	BEAS-2B	↑	220
	NHEK	↓	214
	rat Schwann cells	↓	221
TNFα	rat Schwann cells	↓	218
HGF	rat hepatocytes	↓	222

BME = bovine microvascular endothelial cells; NRK = normal rat kidney cells; Balb C 3T3:

mouse embryonic cell line; T51B: rat liver epithelial cells; NHEK = normal human epidermal keratinocytes; K7: Ni(II)-immortalized human kidney epithelial cells; IL-1α = interleukin 1α; HUVEC = human umbilical vein endothelial cells; BMP = bone morphogenetic protein; MC3T3-E1: osteoblastic cells; NDFß = neu differentiation factor ß; C3H/10T1/2: mouse embryonic fibroblast cell line; BE = normal human bronchial epithelial cells; BEAS-2B = adenovirus 12-SV40 hybrid virus transformed BE cells; ↑: increase; ↓: decrease.

Aim of the study

Disturbance of SMC growth control mechanisms may be seen as a key event in the pathogenesis of atherosclerosis. The research presented in this thesis pertains to the hypothesis that leukocytes play an important role in this pathological process. In particular, the effect of leukocyte-derived growth factors and cytokines on SMC growth control was studied, since the effect of these endogenous factors on for instance GJIC between SMC has not been established previously.

The main objective of this study was to determine whether growth factors and cytokines produced by macrophages and T lymphocytes could influence GJIC between SMC and to provide extensive information about the linkage of growth factors, cytokines and GJIC. We incubated SMC from human umbilical cord arteries with various concentrations of (human) recombinant growth factors and cytokines. Subsequently, GJIC between these SMC was measured. Different exposure times revealed the time course of GJIC modulations upon incubation with growth factors and cytokines (chapter 2 and 3). Interactive effects of these factors on GJIC were studied as well, by adding up to three growth factors and cytokines simultaneously to SMC (chapter 4).

A second important objective of this study was to determine whether macrophages could influence GJIC between co-cultured SMC. In this type of experiments, the complexity of macrophage secretion patterns were reflected more fully. We cultured J774A.1 murine macrophages or human monocyte-macrophages on pore membrane inserts together with human SMC in the Transwell-COL cell culture system. After removal of the inserts containing macrophages, GJIC between the co-cultured SMC was measured (chapter 5).

Chapter 2

Inhibition of Gap Junctional Intercellular Communication between Primary Human Smooth Muscle Cells by Tumor Necrosis Factor α

A. Mensink, L.H.J. de Haan, C.M.M. Lakemond, C.A. Koelman and J.H. Koeman

Based on: Carcinogenesis 16:2063-2067 (1995)

Abstract

Tumor necrosis factor α (TNFα), a pleiotrophic cytokine present in atherosclerotic lesions, caused a dose-dependent and persistent reduction in gap junctional intercellular communication (GJIC) between primary human smooth muscle cells (SMC) *in vitro*. A continuous presence of TNFα was required for this persistent inhibition. Pretreatment of SMC with ascorbic acid, α-tocopherol or glutathione prevented this inhibition of GJIC by TNFα. The persistent blockage of GJIC by continuous exposure to TNFα suggests that TNFα may share some mechanistic similarities with exogenous tumor promoters. Furthermore, this reduction in GJIC by TNFα may provide an additional link between the processes of atherosclerosis and carcinogenesis. The protection afforded by antioxidant compounds suggests a role for active oxygen species in the promotion stage of atherosclerosis.

Introduction

Atherosclerosis, characterized as a focal thickening of the intimal layer in the artery wall, is considered to be the primary cause of mortality in Western societies. Lesion progression is accompanied by the indolent proliferation of smooth muscle cells (SMC) and macrophages (8,11). Ultimately, the atherosclerotic plaque can cause occlusion of the blood vessel, giving rise to myocardial infarction and cerebral haemorrhage.

There are several theories regarding the etiology of atherosclerotic plaques, including the monoclonal hypothesis and the response-to-injury theory. In both theories disturbance of growth control mechanisms is seen as a key event in the pathogenic process. The response-to-injury theory (29) focuses on the interaction between endothelial cells, SMC, monocytes/macrophages and platelets in the lesion, resulting in the autocrine and/or paracrine action of growth factors and cytokines on SMC. The monoclonal hypothesis (30) assumes that the atherosclerotic lesion is a tumor developed out of one single mutated SMC. Experiments demonstrating that human plaque DNA was able to transform NIH 3T3 fibroblasts (46) provided additional evidence for this monoclonal theory. From the monoclonal point of view, the pathogenesis of plaque formation can be divided in distinct stages of initiation and promotion (33), similar to the process of tumorigenesis.

Gap junctional intercellular communication (GJIC) is regarded as an essential mechanism in the control of cell growth (71). Modulation of GJIC is also likely to play an important role in the process of carcinogenesis: *in vitro* and *in vivo* studies provide strong evidence for the involvement of GJIC in tumor promotion (71,72). Moreover, there is evidence that altered GJIC is of importance in the pathogenesis of atherosclerosis as well. Zwijsen *et al.* (57-59) and De Haan *et al.* (60) showed that atherogens, such as oxidized low density lipoprotein, cigarette smoke condensate, oxysterols and aldehydes are able to reduce GJIC between SMC, suggesting that disturbance of gap junctional-mediated growth control by these atherogens might contribute to the SMC proliferation seen in atherosclerotic lesions.

It is likely that this SMC proliferation is also influenced by the action of cytokines and growth factors, which can be produced locally by the macrophages present or by the SMC themselves. Immunohistochemical analysis showed that tumor necrosis factor α (TNFα), a pleiotrophic cytokine, is present in atherosclerotic lesions in elevated amounts as compared with the normal vessel wall (92,94).

In vitro studies show that arterial SMC proliferation is stimulated by TNFα (105,106). TNFα modulates the phenotype of SMC (106) and activates several SMC functions, for instance induction of interleukin 1 production, release of prostaglandin E2,

time-dependent induction of the gene for (2'-5')-oligoadenylate synthetase (98) and induction of alterations of SMC glycosaminoglycans (122).

The present study focuses on the role of TNFα in the modulation of GJIC between primary human SMC, suggesting a role for this cytokine in the promotion stage of atherosclerosis. Furthermore, a first step is taken to elucidate the mechanism of action of this TNFα mediated process.

Materials and methods

Chemicals

Recombinant human TNFα (molecular weight 36 kD; specific activity of the batches $\approx 10^8$ units/mg) was obtained from Genzyme Diagnostics (Cambridge, MA). Eagle's minimum essential medium (modified) with Earle's salts (EMEM) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Fetal calf serum (FCS), gentamicin and fungizone were obtained from Gibco BRL (UK). Dulbecco's 'A' phosphate-buffered saline (PBS) was from Oxoid Ltd. (UK). NADH was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Trypsin 1:250 was from Difco (USA). Sodium pyruvate was from BDH Chemicals Ltd. (UK). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES), ethanol, potassium phosphate, lithium chloride and DL-α-tocopherol were purchased from Merck (Germany). Triton X-100, Lucifer yellow CH, bovine serum albumin (BSA) fraction V, glutathione (GSH) and L-ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture of primary human SMC

Primary human SMC were obtained from arteries of human umbilical cords by an explant technique. In brief, arteries were isolated aseptically. The adventitial layer was carefully removed, whereafter the arterial tissue was cut into small pieces. Explants were incubated in EMEM supplemented with 10% FCS, 50 μg/ml gentamicin and 1.25 μg/ml fungizone in a 37°C, 5% CO_2 humidified atmosphere. Sufficient cell growth to permit subculturing was obtained after 3-4 weeks incubation. Cells displayed the well known 'hills and valleys' pattern after reaching confluence and were characterized by immunohistochemical analysis of SMC α-actin. Experiments were performed on cells in passages 2-4.

Experimental procedure

For measurement of GJIC, SMC were cultured in 35 mm culture dishes (Greiner)

until confluency. Serum containing culture medium was removed, whereafter cells were washed with PBS. EMEM supplemented with antibiotics and 0.1 - 0.5 % BSA was added to the cells. Cells were then incubated with TNFα or PBS as an appropriate blank. Just prior to GJIC measurement, HEPES buffer (pH 7.4) was added to the incubations (final concentration 20 mM) to stabilize the pH during microinjection and fluorescence microscopy. Pretreatment of cells with antioxidants occurred as follows: ascorbic acid in serum-free culture medium containing HEPES was added 2.5 h before exposure of cells to TNFα; GSH and α-tocopherol were added to the cells in serum containing culture medium 24 h and 48 h respectively prior to TNFα exposure. Antioxidant containing culture medium was removed and cells were washed twice with PBS, after which the cells were exposed to 0.5 nM TNFα for 1 h.

Measurement of GJIC

GJIC was determined after microinjection of a 20% Lucifer yellow CH (in 0.33 M lithium chloride) solution in a single cell (223). In each SMC culture at least 20 individual cells were microinjected using a vertical injection system (Olympus Injectoscope IMT-2-syf) (224) with a dye filled capillary glass tip (Clark, Pangbourne, UK). The glass capillary tip was prepared using an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of 1 μm. The Lucifer yellow CH filled cells were checked with phase-contrast and fluorescence microscopy directly after microinjection. Fifteen to twenty minutes after the first injection the number of communicating cells was determined using fluorescence microscopy. The average number of fluorescent cells was calculated for each incubation. The average number of communicating cells in control incubations was taken as 100% GJIC. Photographs were taken using an Olympus OM 4 Ti camera using an injectoscope. Each experiment was performed in duplicate. At least three independent tests were done, except for the time curves, where two independent tests were performed. At least one test was performed in a 'double blind' way.

Cytotoxicity assay

SMC were grown in six-well tissue culture plates (Costar Europe Ltd.). When confluent, cells were (pre)incubated as described above. After treatment(s), media were taken from the wells and centrifuged. Cells were scraped from the bottom of the wells after addition of 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.5, followed by a sonification step in ice water (5 min, Sonorex RK 100) and a centrifugation step. Lactate dehydrogenase (LDH) activity was measured in the supernatants (225) and LDH leakage was calculated. Each experiment was performed at least in duplicate and at least two independent tests were done.

Statistics

Statistical analyses of the data were performed using Students *t*-test ($P < 0.05$).

Results

Smooth muscle cell GJIC

Primary human SMC display a profound GJIC (Fig. 2.1). In typical experiments Lucifer yellow spread over an average of 20 - 100 cells (depending on cell density at confluency) in control incubations. In most experiments a control value of ~40 communicating cells was observed.

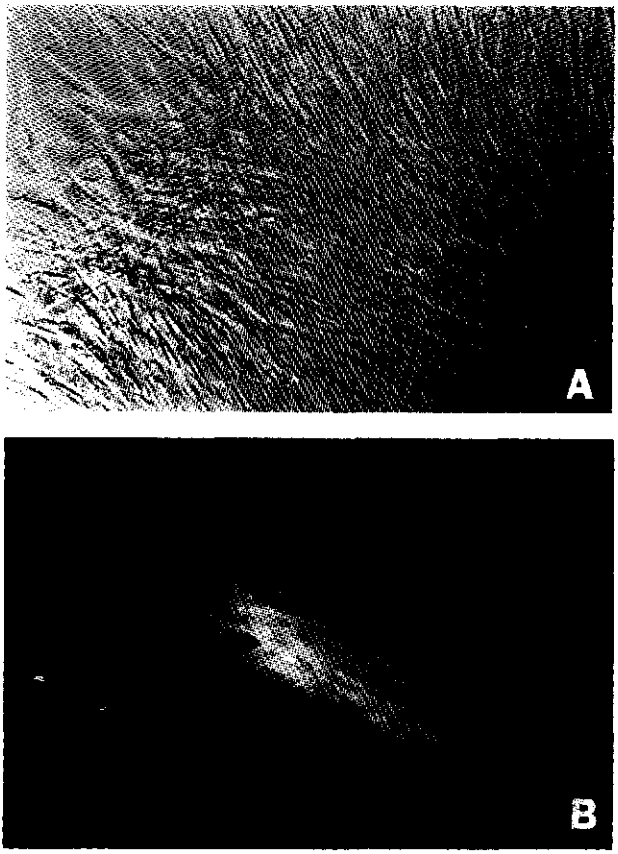

Fig. 2.1. Photographs showing Lucifer yellow transfer in confluent primary human SMC. **(A)** Phase contrast graph of SMC. **(B)** Fluorescence in the same field after injection of one SMC with Lucifer yellow. Original magnification x200.

GJIC between primary human SMC upon 1 h exposure to TNFα

Figure 2.2 illustrates the dose-dependent decrease in GJIC between primary human SMC exposed to 0.05 - 2.5 nM TNFα. A significant, 20% reduction in GJIC was already observed at exposure of these cells to 0.05 nM TNFα. At 0.3 nM TNFα a maximum of 40% inhibition of GJIC was reached and inhibition remained at this level up to a concentration of 2.5 nM.

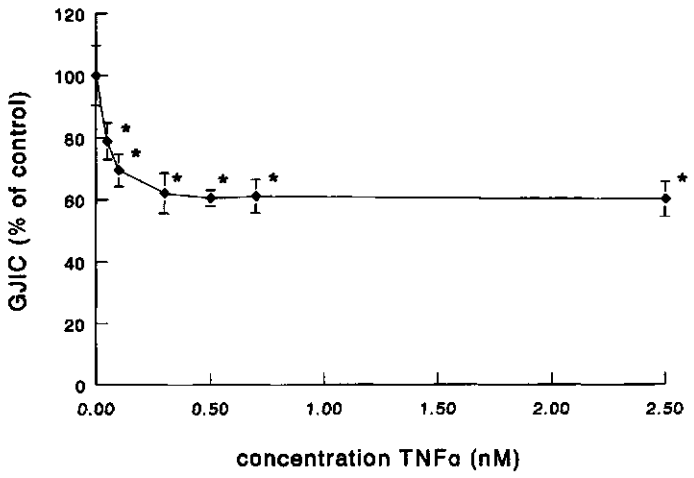

Fig. 2.2. GJIC between human SMC after exposure for 1 h to the indicated concentrations of TNFα. All values are mean ± SD. * : Significantly different from control value.

GJIC between primary human SMC upon exposure to TNFα for different periods of time

A significant inhibition of GJIC was already observed 1 h after exposure to TNFα (Fig. 2.3). The inhibition of GJIC upon exposure to TNFα persisted until the last time point in these series of experiments (24 h). Upon incubation with 0.05 nM TNFα, GJIC was reduced to 75% of control incubations. Incubation with 0.5 nM TNFα also resulted in a persistent inhibition of GJIC, to a level of 65% of control. To investigate whether the persistent inhibition of GJIC required the continuous presence of TNFα, experiments were performed whereby a 1 h exposure to TNFα was followed by replacement of this exposure medium by control medium (Fig. 2.4). Upon removal of TNFα from the culture medium, the inhibited response of GJIC was restored to control values within 30 min.

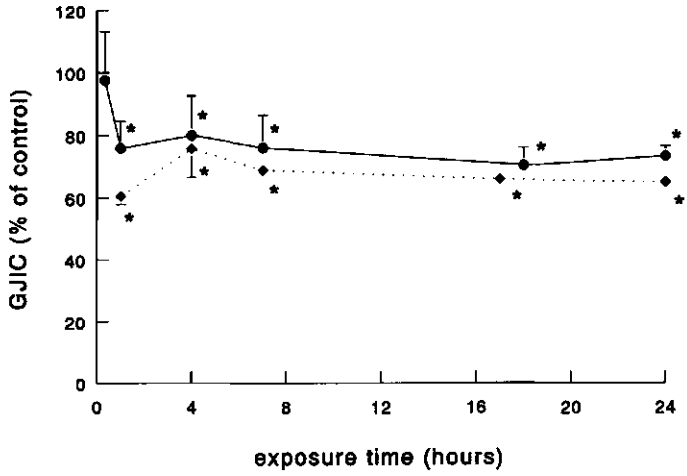

Fig. 2.3. GJIC between human SMC after exposure to TNFα for different periods of time. Circles, 0.05 nM TNFα; diamond, 0.5 nM TNFα. All values are mean ± SD. SD for control incubations varied between 3.6 and 8.7%. * : Significantly different from control value.

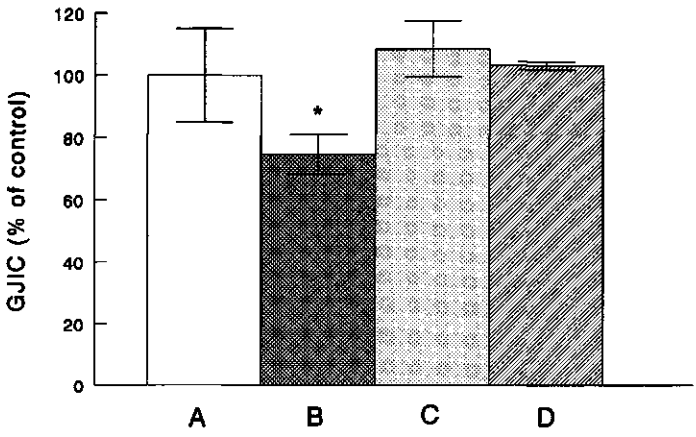

Fig. 2.4. GJIC between human SMC. **(A)** control incubation; **(B)** 1 h 0.5 nM TNFα; **(C)** 1 h 0.5 nM TNFα followed by replacement of exposure medium by control medium for 1 h; **(D)** 1 h 0.5 nM TNFα followed by replacement of exposure medium by control medium for 30 min. All values are mean ± SD. * : Significantly different from control value.

Effects of antioxidants on GJIC between primary human SMC upon exposure to TNFα

Pretreatment of primary human SMC with 1 or 10 μM α-tocopherol, 10 or 50 μM ascorbic acid, or 1 mM GSH prevented the inhibition of GJIC upon exposure to 0.5 nM TNFα (Fig. 2.5). None of the GJIC values from antioxidant pre-incubations was significantly different from the control value, which was set at 100%.

Cytotoxicity

It is reported that TNFα can damage plasma membranes under certain circumstances. This membrane permeabilization can be a relatively fast event, occurring within a few hours after TNFα exposure (226). To determine whether the observed reduction in SMC GJIC was influenced by loss of membrane integrity, LDH activity was measured in culture media and cell homogenates. No membrane cytotoxicity was observed in SMC cultures treated with 0.5 and 2.5 nM TNFα for 1 or 24 h. Values of LDH activity in cell homogenates after exposure to TNFα varied between 95 and 102 % of the control values (data not shown). Also, no cytotoxic effects were seen at concentrations and treatment times of the antioxidants used in the experiments (data not shown). The combination of antioxidant treatment and incubation with 0.5 nM TNFα did not cause membrane leakage either. Furthermore, no morphological changes of SMC were seen in our experiments.

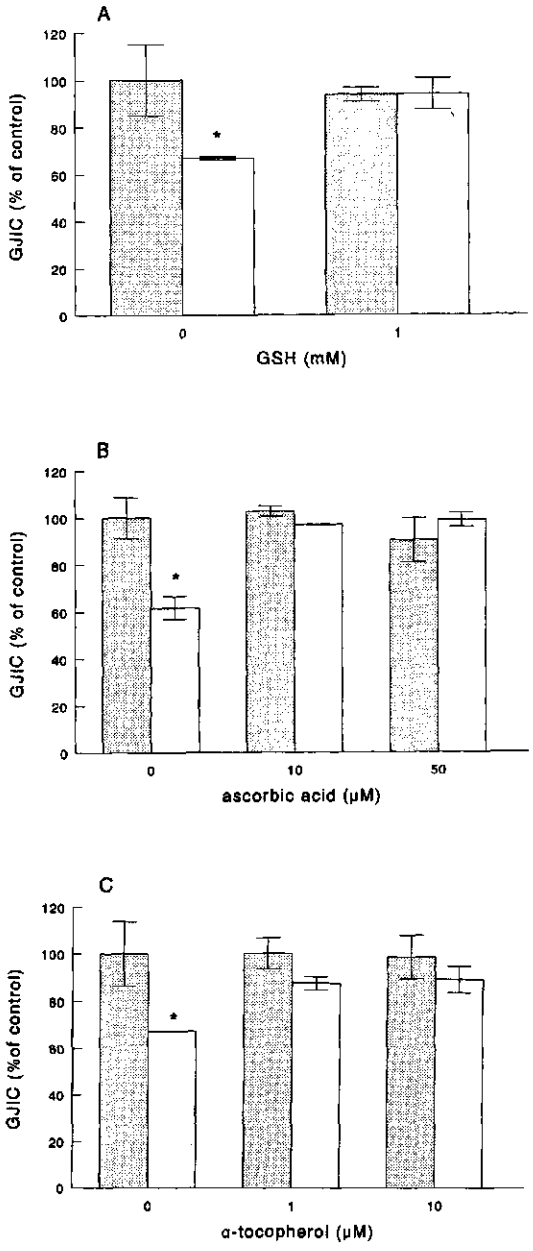

Fig. 2.5. GJIC between human SMC after pretreatment with GSH **(A)**, ascorbic acid **(B)** or α-tocopherol **(C)**. Dotted bars represent control incubations; open bars represent TNFα exposures. All values are mean ± SD. * : Significantly different from control value.

Discussion

The present study clearly demonstrates that TNFα is able to significantly reduce GJIC between primary human SMC. The results also indicate that free radicals may be responsible for the observed effect of TNFα on GJIC.

Our results show a resemblance to those reported by Fujiki & Suganuma (227) and Komori *et al.* (228), who demonstrated that TNFα acts as a tumor promoter in the BALB / 3T3 assay.

The reduction in GJIC between primary human SMC occurred in a dose-dependent way, reaching ~40% inhibition at a dose of 0.3 nM TNFα. Serum values of TNFα are reported to be of the order of 10 - 65 pg/ml (92,229). Assuming a molecular weight of 36 kD for TNFα, this may correspond to a concentration of 0.3 - 2.0 pM. Rus *et al.* (92) reported that the TNFα concentration in the arterial wall was ~200 times higher than in serum, which may result in concentrations of 0.06 - 0.40 nM. This estimated target tissue concentration is within the range of the inhibitory response of TNFα on GJIC observed in our *in vitro* study. This may suggest that inhibition of GJIC between SMC can also occur *in vivo*.

Inhibition of GJIC caused by TNFα lasts for at least 24 h. This persistent reduction in GJIC suggests a strong atherogenic potential of the cytokine TNFα. However, on removing TNFα from the culture medium the inhibited response of GJIC was quickly restored. This indicates that a continuous presence of TNFα is required to obtain a persistent inhibition. Such a continuous presence of TNFα may be achieved through local synthesis and accumulation of TNFα in the arterial wall, as was suggested by Rus *et al.* (92). Immunohistochemical analysis and *in situ* hybridization studies clearly show that macrophages and SMC can serve as sources for TNFα in the lesions (93,94).

The biological consequence of this 40% reduction in GJIC by TNFα is not easily given, since this value was obtained in an *in vitro* assay in which, for instance, the arrangement of SMC in culture dishes will deviate from their organization in the vascular wall. The extrapolation is difficult to make for known tumor promoters as well. The inhibition in GJIC caused by TNFα, a body's own substance, is somewhat lower, but comparable with the 50 - 60% inhibition caused by well known xenobiotic tumor promoters like coplanar polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (230). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) inhibits GJIC *in vitro* almost completely. However, this inhibition of cell communication by TPA is transient and is restored within 24 h (231), while on the other hand both PCB/dioxin and TNFα inhibit GJIC for a prolonged period of time.

The rapid occurrence of GJIC inhibition (within 1 h) and the fast reversal of this

inhibition following removal of TNFα from the culture medium points towards a rapid membrane-linked mechanism of action. Several cell type-dependent post-receptor signal transducing pathways are known to be affected by TNFα (90). TNFα signalling mechanisms include activation of phospholipase A_2, stimulation of phospholipase C, stimulation of phospholipase D, protein phosphorylation, G protein activation, activation of protein kinase C and enhancement of cAMP levels (232). All these processes are known to affect GJIC.

Furthermore, several lines of evidence indicate that reactive oxygen formation and/or production of nitric oxide are involved in TNFα mediated processes (90). The present study indicates that free radical production by SMC might play a role in the observed reduction in GJIC caused by TNFα, since pretreatment of these cells with ascorbic acid, α-tocopherol or GSH prevented inhibition of GJIC. The observed prevention of inhibition of GJIC by antioxidants has been previously reported in studies with classical chemical tumor promoting agents (233). Furthermore, a correlation between GJIC and GSH levels in cells has been reported (234). Further research, currently in progress, is aimed at revealing whether free radicals are actually formed by SMC on exposure to TNFα. In addition, the radical species involved and their mode of action with regard to the inhibition of GJIC between SMC will be determined. However, other mechanisms of GJIC regulation cannot be ruled out. For instance, α-tocopherol is able to inhibit protein kinase C translocation and activity in rat SMC (235).

The results of the present study may contribute to the increasing data supporting the view that radicals are important in tumor promotion (236). For instance, it is known that active oxygen can act as a tumor promoter in cell transformation assays (237).

In studying atherosclerosis much attention has been given to the (per)oxidation of low density lipoprotein components (238), causing amongst other things endothelial damage, foam cell formation, SMC proliferation and immune system activation. Free radicals can also directly activate SMC growth (239). Thus, active oxygen seems to be involved in many stages of the pathogenic process. Our study adds a new concept to the role of active oxygen in the process of atherosclerosis; namely their possible role in inhibition of GJIC and thus in the promotion stage of this disease.

In conclusion, the persistent blockage of GJIC by TNFα suggests that TNFα may act as an endogenous 'promoter' on human SMC. Furthermore, this reduction in GJIC by TNFα may provide an additional link between the processes of atherosclerosis and carcinogenesis, a connection previously suggested by Zwijsen *et al.* (162,240).

Acknowledgements

The authors wish to thank Dr Abraham Brouwer and Professor Dr Wim M.F. Jongen for critically reading the manuscript and helpful discussions, Dr G. Henrita van Zanten for supplying some of the SMC used in these experiments when we were temporally deprived of these cells and Drs C.W.D.A. Klapwijk and his coworkers for their cooperation.

Chapter 3
Modulation of Intercellular Communication between Smooth Muscle Cells by Growth Factors and Cytokines

A. Mensink, A. Brouwer, E.H. van den Burg, S. Geurts, W.M.F. Jongen, C.M.M. Lakemond, I. Meijerman and T. van der Wijk
Based on: Eur. J. Pharmacol. 310:73-81 (1996)

Abstract

We recently reported that tumor necrosis factor α (TNFα) is able to cause a dose-dependent and persistent reduction in gap junctional intercellular communication (GJIC) between primary human smooth muscle cells (SMC). In order to study whether this observed persistent reduction in GJIC is a unique feature for TNFα, the present study focuses on the effects of other growth factors and cytokines on GJIC. Platelet-derived growth factor AA and BB (PDGF-AA, PDGF-BB), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6) and interferon-γ (IFN-γ) were able to modulate GJIC between primary human SMC *in vitro*. However, our results demonstrate that the magnitude and nature of the observed effects are growth factor- and cytokine-specific. PDGF-AA, PDGF-BB and IL-6 caused a transient reduction in GJIC, while bFGF induced a transient increase in GJIC. IFN-γ was shown to be capable of causing a persistent reduction in GJIC. In addition, PDGF-AA, PDGF-BB, bFGF, IL-6, IFN-γ and TNFα all stimulated SMC proliferation. These observations suggest a more complex relationship between modulation of GJIC and cell proliferation than current hypotheses imply. The implications of the observed effects of growth factors and cytokines on GJIC between SMC in relation to the process of atherosclerosis are discussed.

Introduction

Atherosclerosis is a pathophysiological phenomenon with a slow progression giving rise to myocardial and cerebral infarctions. Atherosclerotic lesions ('plaques') are characterized as focal thickenings of the intimal layer of the artery wall. Lesion progression is accompanied by infiltration of monocytes and lymphocytes, proliferation of smooth muscle cells (SMC), accumulation of intra- and extracellular lipids and synthesis of extracellular matrix components.

Regarding the etiology of atherosclerosis, both the response-to-injury theory and the monoclonal hypothesis focus on disturbance of growth control mechanisms as the key event in atherogenesis. Consequently, both theories assume an important modulatory role for growth factors and cytokines in the pathogenesis of the disease. The response-to-injury theory (29) supposes that growth factors and cytokines produced by cell types present in the lesions, act in an autocrine and/or paracrine manner on SMC proliferation. The monoclonal hypothesis (33) parallels atherogenesis with tumorigenesis, thereby dividing the pathogenesis of plaque formation in stages of initiation and promotion.

Hybridization and immunohistochemical studies demonstrated that platelet-derived growth factor AA and BB (PDGF-AA, PDGF-BB), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) are present in human atherosclerotic plaques (4,94,141,142,194). It is not completely clear whether the amount of all these growth modulating substances is elevated in plaques when compared with the nonatherosclerotic vessel wall. bFGF, IL-6, PDGF and TNFα are able to induce SMC proliferation (241). It has been reported that IFN-γ either stimulates (126) or suppresses (128,129) SMC proliferation, probably depending on the culture conditions for these cells.

In vitro and *in vivo* studies provide evidence for the involvement of modulation of gap junctional intercellular communication (GJIC) in the process of tumor promotion (71). Altered GJIC seems to be important in the pathogenesis of atherosclerosis as well. Atherogens like oxidized low density lipoprotein, cigarette smoke condensate, oxysterols and aldehydes are able to reduce GJIC between SMC (57-60).

Previous work (242) demonstrated that TNFα is able to cause a dose-dependent and persistent reduction in GJIC between primary human SMC, suggesting that TNFα may act as an endogenous 'promoter' on human SMC. In order to study whether this observed persistent reduction in GJIC between SMC is a unique feature for TNFα or a more common event in growth factor or cytokine action on these cells, the present study focuses on the effects of PDGF-AA and PDGF-BB, bFGF, IL-6 and IFN-γ on GJIC between primary human SMC. Furthermore, we have studied the effect of these growth

modulating peptides and TNFα on SMC proliferation in our cell culture system, in order to obtain more knowledge about the relationship between GJIC and cell proliferation.

Materials and methods

Chemicals

Recombinant human TNFα (molecular weight 36 kD; specific activity 10^8 U/mg), recombinant human IL-6 (molecular weight ≈26 kD; specific activity 1.8 x 10^8 and 1.8 x 10^9 U/mg) and recombinant human IFN-γ (molecular weight 34 kD; specific activity 2.5 x 10^7 U/mg) were obtained from Genzyme Diagnostics (Cambridge, MA, USA). PDGF-AA, PDGF-BB and bFGF were obtained from Genzyme Diagnostics (Cambridge, MA, USA; molecular weight PDGF 26 kD, molecular weight bFGF 17.5 kD) and from Gibco BRL (Paisley, UK, molecular weight PDGF 30 kD, molecular weight bFGF 17 kD). Cell proliferation kits based on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cleavage by mitochondrial dehydrogenases and NADH were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Eagle's minimum essential medium (modified) with Earle's salts (EMEM) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Fetal calf serum (FCS), gentamicin and fungizone were obtained from Gibco BRL (Paisley, UK). Dulbecco's 'A' phosphate-buffered saline (PBS) was from Oxoid Ltd. (UK). Trypsin 1:250 was from Difco (USA). Sodium pyruvate was from BDH Chemicals (UK). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), potassium phosphate, lithium chloride and acetic acid were purchased from Merck (Darmstadt, Germany). Triton X-100, Lucifer yellow CH, and bovine serum albumin (BSA) fraction V were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Culture of primary human SMC

Primary human SMC were isolated from arteries of human umbilical cords by an explant technique. After careful removal of the adventitial layer, the arterial tissue was cut into small pieces. Explants were incubated in EMEM supplemented with 10% FCS, 50 μg/ml gentamicin and 1.25 μg/ml fungizone in a 37°C, 5% CO_2 humidified atmosphere. Cells were allowed to grow for 3-4 weeks until subculturing. Cells displayed the well known 'hills and valleys' pattern after reaching confluence (243). Experiments were performed on cells in passage 2-5.

Experimental procedure

For measurement of GJIC, SMC were grown in 35 mm culture dishes (Greiner

B.V., Alphen a/d Rijn, Netherlands) until confluency. Serum containing culture medium was removed whereafter cells were washed with PBS. EMEM supplemented with antibiotics and 0.1 - 0.5 % BSA was added to the cells. Cells were then incubated with the growth modulating factors or with either PBS (in the case of IL-6, IFN-γ, bFGF) or acetic acid (in the case of PDGF) as appropriate blanks. Just prior to GJIC measurement, HEPES buffer (pH 7.4) was added to the incubations (final concentration 20 mM) to stabilize the pH during microinjection and fluorescence microscopy.

Measurement of GJIC

GJIC was determined after microinjection of a 20% Lucifer yellow CH (in 0.33 M lithium chloride) solution in a single cell (223). In each SMC culture at least 20 individual cells were microinjected using a vertical injection system (Olympus Injectoscope IMT-2-syf) (224) with a dye filled capillary glass tip (Clark, Pangbourne, UK). The capillary glass tip was prepared using an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of 1 μm. The Lucifer yellow CH filled cells were checked with phase-contrast and fluorescence microscopy directly after microinjection. 15 - 20 min after the first injection the number of communicating cells was determined using fluorescence microscopy. The average number of fluorescent cells was calculated for each incubation. The average number of communicating cells in control incubations was taken as 100% GJIC. Each experiment was performed in duplicate. At least three independent tests were done. Data presented in figures 3.1 - 3.4 are the average values of these tests.

Cell proliferation assays

5000 - 10000 SMC (n=8) were plated in serum containing culture medium in 96 wells microtiter plates (tissue culture grade, Greiner B.V., Alphen a/d Rijn, Netherlands); 100 μl medium per well. After 24 h, the culture medium was removed and the cells were washed with PBS. EMEM supplemented with antibiotics and 0.5% BSA was added to the cells. Cells were then incubated with the growth modulating factors or with either PBS or acetic acid as appropriate blanks for 24 h, whereafter cell proliferation was measured. After incubation with growth modulating factors, SMC cells were incubated with MTT reagent according to the instructions of the manufacturer. The MTT colorimetric assay is based on cleavage of the yellow tetrazolium salt MTT to formazan dye by dehydrogenase activity in active mitochondria of living cells. The formazan dye formed is quantified using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA). An increase in number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample, which correlates to the amount of formazan formed as monitored by the absorbance at 595 nm.

Cytotoxicity assay

SMC were grown in six-well tissue culture plates (Costar Europe Ltd.). When confluent, cells were incubated as for GJIC measurement. After treatment, media were taken from the wells and were centrifuged. Cells were scraped from the bottom of the wells after addition of 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.5, followed by a sonification step in ice water (5 min, Sonorex RK 100 (Bandelin GmbH, Berlin, Germany)) and a centrifugation step. Lactate dehydrogenase (LDH) activity was measured in the supernatants (225) and LDH leakage was calculated. Each experiment was performed at least in duplicate and at least two independent tests were done.

Statistics

Statistical analyses of the data were performed using Students *t*-test ($P < 0.05$).

Results

GJIC between SMC

Primary human SMC display a distinct GJIC. In typical experiments Lucifer yellow spread over an average of 15-80 cells (probably depending on cell density at confluency) in control incubations. In most experiments a control value of ~40 communicating cells was observed. Within separate experiments, standard deviations for duplicate incubations were generally small. The standard deviations for control incubations and growth factor or cytokine incubations were quite similar within experiments. In experiments with IFN-γ, bFGF and IL-6 the average standard deviations within single experiments were 3 - 5%. In experiments with PDGF results were more variable; standard deviations were a few percent higher than in the other experiments. Standard deviation values in the order of 10% were seen in a number of cases in separate PDGF experiments.

PDGF

PDGF-AA and PDGF-BB reduce GJIC between primary human SMC in a similar way. In 1 h incubations, GJIC is reduced to ~ 80% of control value at 0.1 nM PDGF (Fig. 3.1 A,B). Higher concentrations PDGF-AA and PDGF-BB resulted in a stronger decrease in GJIC, with a maximal inhibition of 35-40%, reached at 0.5 - 0.7 nM PDGF. The observed inhibition in GJIC is transient for both PDGF isoforms with a maximum inhibition after 1 h incubation followed by a return to almost control values within 24 h (Fig. 3.2 A,B). Replacement of PDGF containing culture medium with medium contai-

ning fresh PDGF after 3 h during an exposure time of 4 h did not give rise to different experimental outcomes for both PDGF-AA and PDGF-BB isoforms (data not shown). Thus, the transient nature of this GJIC inhibition upon incubation with PDGF isoforms appeared not to be caused by a depletion of PDGF in the culture medium.

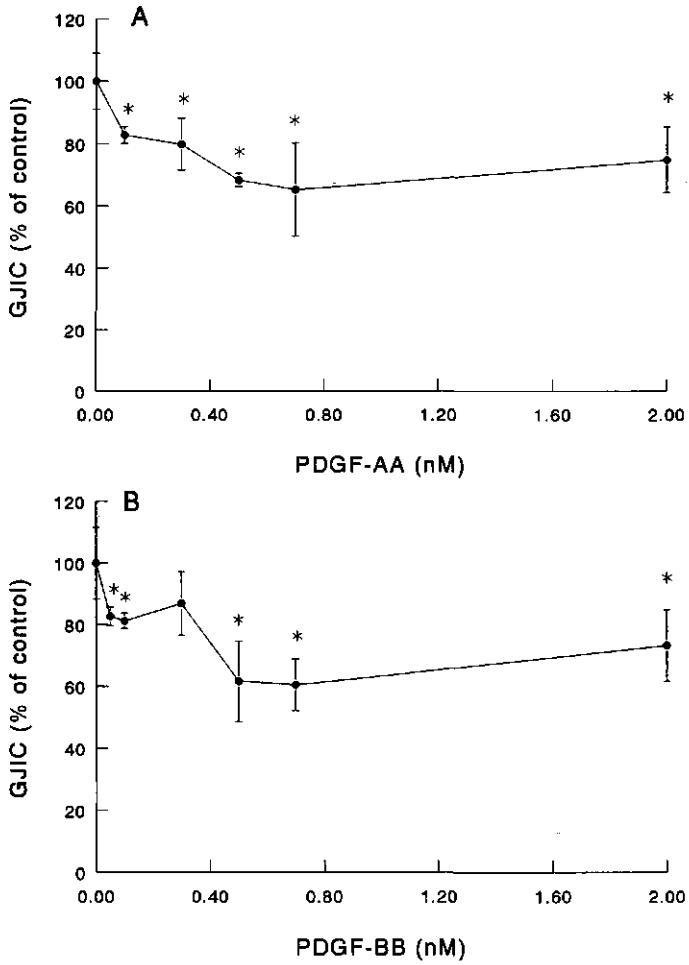

Fig. 3.1. Dose-dependent inhibition of GJIC between human SMC by PDGF-AA (**A**) or PDGF-BB (**B**). Exposure time was 1 h to the indicated concentrations of PDGF-AA or PDGF-BB. All values are mean ± SD. * : Significantly different from control value.

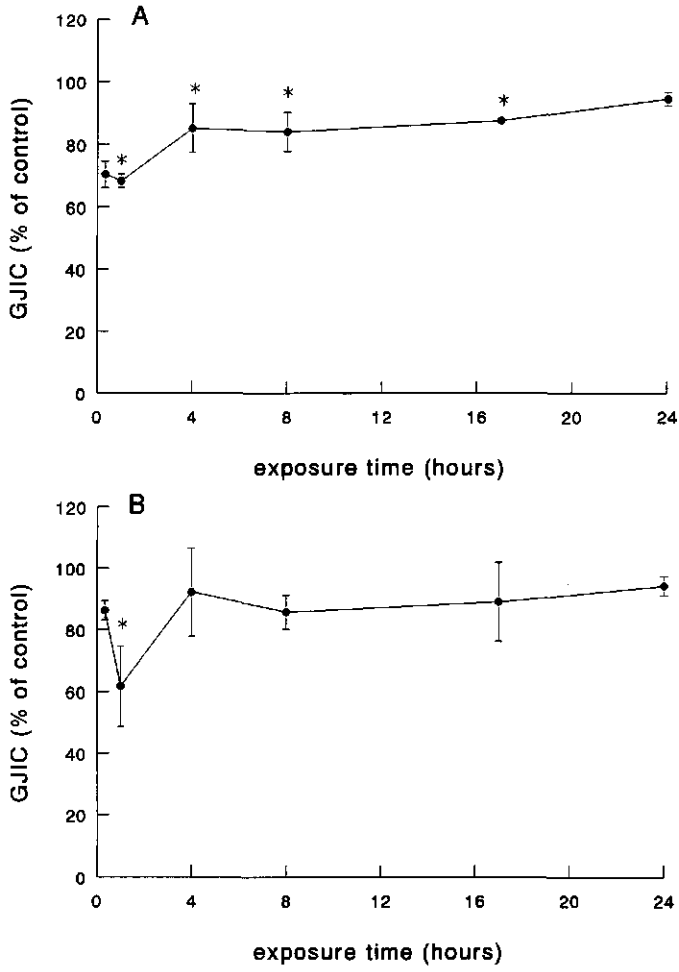

Fig. 3.2. Time-dependent inhibition of GJIC between human SMC by PDGF-AA **(A)** or PDGF-BB **(B)**. Exposure concentration was 0.5 nM PDGF-AA or 0.5 nM PDGF-BB for the indicated periods of time. All values are mean ± SD. Average SD for control incubations was 9.0% **(A)** and 11.7% **(B)**. * : Significantly different from control value.

IL-6

One hour incubations of confluent SMC cultures with 0.5 nM IL-6 did not result in a consistent reduction in GJIC between these cells (Fig. 3.3 A). Incubation with 2.5 and 5.0 nM IL-6 for 1 h caused a small but significant reduction in GJIC of 13 ± 4.5

and 17 ± 6.0 %, respectively. However, when SMC were incubated for a longer period of time with 0.5 nM IL-6, a significant reduction in GJIC was obtained at 7 and 9 h of exposure (Fig. 3.3 A). At these time points, GJIC was inhibited to ~80% of control incubations. At 17 and 24 h of incubation with 0.5 nM IL-6, GJIC was restored to control values (Fig. 3.3 A). Again, the transient nature of this GJIC inhibition by IL-6 appeared not to be a consequence of a depletion of the cytokine in the culture medium, since a 17 h incubation time resulted in the same GJIC value as a 10 + 7 h incubation with refreshment of IL-6 containing culture medium after 10 h of incubation (data not shown).

IFN-γ

Upon addition of 0.5 nM IFN-γ to SMC, GJIC is reduced to ~80% of control incubations (Fig. 3.3 B). This reduction in GJIC occurred within 1 h and lasted for at least 24 h. When 2.0 or 3.5 nM IFN-γ were added to the SMC for 1 h, GJIC was not further reduced i.e. 20 ± 0.7 and 18 ± 1.4% inhibition respectively.

bFGF

Addition of 0.5 nM bFGF to SMC resulted in a significant increase in GJIC to 123% of control incubations at 24 h of exposure only (Fig. 3.4 A). At 30 h incubation, GJIC returned to control value, irrespective of addition of fresh bFGF containing culture medium after 6 h incubation (data not shown). Incubation of SMC with 2.0 and 4.0 nM bFGF for 24 h resulted in a further increase in GJIC; with a doubling of the number of communicating SMC at 4.0 nM (Fig. 3.4 B).

Cytotoxicity

To determine whether the observed effect on SMC GJIC was influenced by loss of membrane integrity upon incubation with growth factors or cytokines, LDH activity was measured in culture media and cell homogenates. No membrane cytotoxicity was observed in SMC cultures treated for 24 h with 0.5 or 2.5 nM IL-6, 0.5 or 0.7 nM PDGF-AA or PDGF-BB, or 0.5 nM IFN-γ (data not shown). Treatment of cells for 30 h with 0.5 nM bFGF did not cause membrane cytotoxicity either. Thus, these studies on GJIC were done at noncytotoxic concentrations.

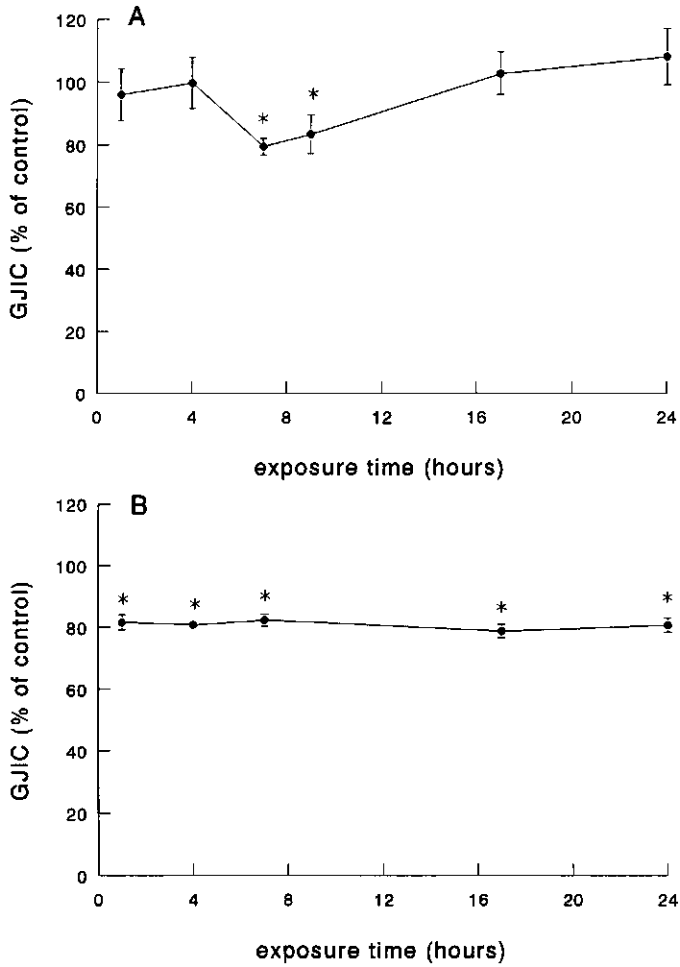

Fig. 3.3. GJIC between human SMC upon incubation with 0.5 nM IL-6 (**A**), or 0.5 nM IFN-γ (**B**) for the indicated periods of time. All values are mean ± SD. Average SD for control incubations was 3.2% (**A**) and 2.8% (**B**). * : Significantly different from control value.

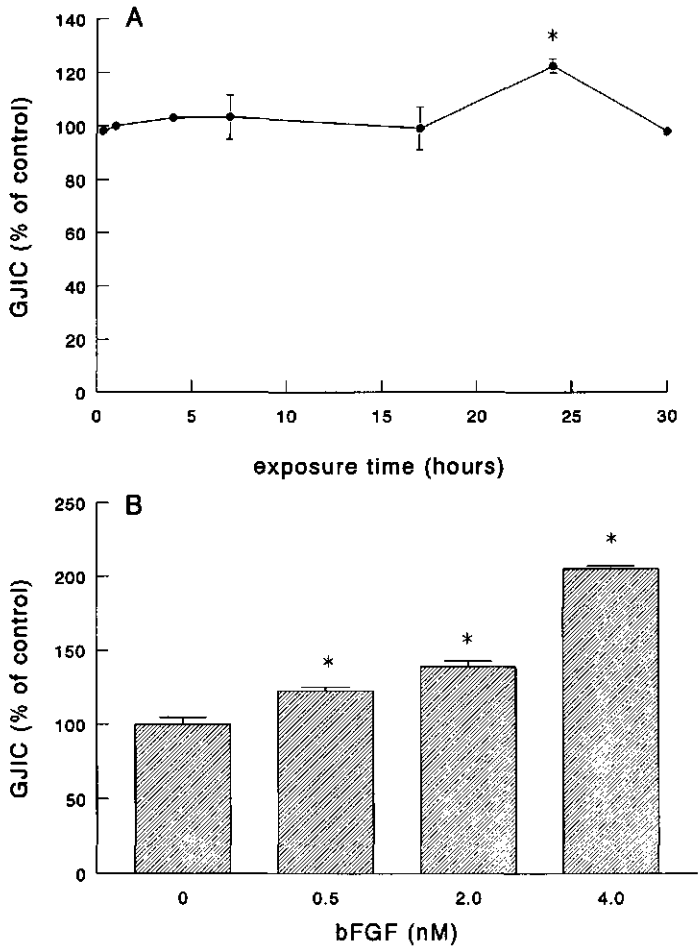

Fig. 3.4. GJIC between human SMC upon incubation with 0.5 nM bFGF for the indicated periods of time (**A**) or after exposure for 24 h to the indicated concentrations of bFGF (**B**). All values are mean ± SD. Average SD for control incubations was 4.8% (**A**). * : Significantly different from control value.

SMC proliferation

In a number of experiments, PDGF-AA, PDGF-BB, IL-6, bFGF, TNFα and IFN-γ were able to stimulate SMC proliferation. In table 3.1 maximal absorbance values (percentages of control) are given for the incubations of SMC with growth factors and cytokines. However, a considerable inter-experimental variance was observed (data not

shown). In some 24 h incubations with growth factors and cytokines, no significant cell proliferation was observed compared to control incubations. In some other experiments, PDGF-AA and PDGF-BB could not induce cell proliferation, whereas the other growth modulating factors did stimulate SMC growth. Furthermore, the magnitude of the proliferative response varied strongly per experiment. In some experiments, TNFα and IFN-γ were the most potent factors tested; in other experiments the tested growth factors and cytokines seemed to be more or less equally potent. Experiments in which SMC were incubated for 72 h with the growth modulating factors also resulted in the above discussed forms of inter-experimental variance (data not shown). In our cell culture system, we never observed a suppression of SMC growth upon incubation with IFN-γ.

Table 3.1.
Maximal absorbance values of SMC incubtions with growth factors and cytokines as measured in the MTT test after incubation with these factors for 24 h

Growth factor / cytokine	Absorbance 595 nm (% of control)
PDGF-AA	118 ± 13 [a]
PDGF-BB	132 ± 17 [a]
IL-6	109 ± 9 [a]
bFGF	107 ± 9 [a]
TNFα	140 ± 20 [a]
IFN-γ	144 ± 19 [a]

Values are mean ± SD (n=8). SD for control incubations varied from 2 to 8%.
[a]: Significantly different from control value.

Discussion

The present study demonstrates that the growth modulating factors PDGF-AA, PDGF-BB, IL-6, bFGF and IFN-γ are able to modulate GJIC between primary human SMC. In the case of PDGF-AA, PDGF-BB and IL-6, a transient reduction in GJIC is observed. On the contrary, a transient increase in GJIC is seen in SMC incubated with bFGF. Furthermore, IFN-γ is shown to be a growth modulating factor capable of causing a persistent reduction in GJIC (>24 h).

Time course effects of IFN-γ show a resemblance with the effects of TNFα on GJIC between human SMC (242). The ~20% inhibition in GJIC caused by IFN-γ is,

however, lower than the ~40% inhibition caused by TNFα, but both factors cause a persistent reduction in GJIC for at least 24 h.

Maldonado *et al.* (211) reported that PDGF (isoform not mentioned) reduced GJIC at 20 min after growth factor application with 0 - 37%, depending on the cell type used. In a dose-response curve at 20 min after addition of PDGF, they observed a 37% inhibition of GJIC between BalbC 3T3 cells upon incubation with 0.6 nM PDGF. This corresponds well with the 35 - 40% inhibition in GJIC between SMC seen in our experiments upon incubation with 0.5 - 0.7 nM PDGF. Pelletier and Boynton (219) demonstrated however, that 0.3 nM PDGF-BB inhibits GJIC between C3H/10T1/2 cells almost completely after 40 min incubation. The transient nature of GJIC inhibition observed in our experiments upon PDGF exposure of SMC, shows a resemblance with the fast recovery of GJIC (within 70 min) between C3H/10T1/2 cells exposed to PDGF-BB, as described by these authors.

The GJIC inhibitory activity of IL-6 appeared to be smaller than was observed for PDGF isoforms. In addition, time course experiments demonstrated a slower response of GJIC inhibition to IL-6 treatment. Only when high concentrations (2.5 or 5.0 nM) of IL-6 were added to the cells, a quick response of GJIC inhibition within 1 h of exposure was observed. The slow response of GJIC inhibition at 0.5 nM IL-6 suggests that IL-6 may have an 'indirect' effect on GJIC as well, perhaps by changing the levels of other growth modulating factors in the SMC culture. For instance, it is known that IL-6 is able to stimulate PDGF production in SMC from rat aorta (148).

In the present study only bFGF stimulated GJIC between SMC. bFGF appeared to be a potent modulator of GJIC in our study with a doubling of the number of communicating cells after 24 h of incubation with 4.0 nM bFGF. This result is comparable with results of Pepper and Meda (207) who demonstrated an increase in GJIC between endothelial cells upon incubation with bFGF.

Our results demonstrate that the modulation of GJIC between SMC is growth factor and cytokine-specific. The reason for the different modulatory effects on GJIC by growth factors and cytokines is unknown, but may be associated with differences in signal transduction mechanisms. The signal transduction pathway after PDGF receptor activation on SMC is relatively well known. Inositol 1,4,5-triphosphate release, diacylglycerol production, calcium mobilization, activation of protein kinase C and changes in intracellular pH have been reported (166,244,245), processes which may modulate GJIC by affecting the permeability of gap junction channels. Pelletier and Boynton (219) observed that inhibition of GJIC by PDGF-BB was dissociable from the PDGF receptor tyrosine kinase activity in C3H/10T1/2 cells. Furthermore, they found that PDGF treatment of these cells resulted in phosphorylation of the connexin 43 (Cx43) protein. Growth factors

may regulate GJIC at the level of connexin transcription, mRNA stability, translation and post-translational processing as well: bFGF treatment of endothelial cells resulted in an increase in Cx43 expression (207). Moreover, Kardami *et al.* (246) have shown that bFGF-like peptides are an integral part of, or exist in close association with gap junctions and may thus modulate GJIC.

The biological consequences of the modulation of GJIC by PDGF-AA, PDGF-BB, IL-6, IFN-γ and bFGF are not easily explainable. Extrapolation from these *in vitro* data to the *in vivo* situation is difficult, since in the *in vitro* assay, for instance, the arrangement of SMC will deviate from the organization of SMC in the vascular wall. Furthermore, primary human SMC from umbilical cord arteries were used in our experiments, which may differ from more adult SMC in atherosclerotic plaques. Both the percentage of increase or decrease in GJIC and the duration of the effect caused by these growth modulating factors may be of importance in determining the relevance for the process of atherogenesis. Growth modulating factors like IFN-γ and TNFα, that cause a persistent reduction in GJIC, are more likely to have impact on the disturbance of normal SMC proliferation than growth factors and cytokines which modulate GJIC only temporarily. However, growth factors and cytokines that cause only a temporal modulation of GJIC may be continuously present and the development of atherosclerotic lesions may take many years.

In agreement with many reports in literature, PDGF-AA, PDGF-BB, IL-6, IFN-γ, bFGF and TNFα all stimulated SMC proliferation in our hands using the MTT test. However, the mitogenic responses of the SMC varied considerably in experiments. This heterogeneity in SMC response with respect to cell proliferation has been reported before (245,247-251). Differences between 'SMC batches' may stem from their derivation from different individuals, variations in cell passage numbers and the possible interchange between the synthetic, secretory phenotype in which cells experience growth and proliferation, and the contractile, nonproliferating phenotype. Hall *et al.* (249) suggest that the heterogeneity in SMC cultures may also represent a heterogeneity of vascular SMC *in vivo*. This heterogeneity *in vivo* may reflect a specialization of function related to the location of SMC in the vascular tissue. These differences in SMC batches may result in differences in for instance SMC size, saturation density, synthesis of matrix components, cell surface receptor number and proliferation rate. Also, it is not unlikely that cell density influences the SMC proliferative response.

Both *in vivo* and *in vitro* studies (77) demonstrated cell cycle-related changes in GJIC. The growth state of cells may influence junctional sensitivity (252). Accordingly, several reports suggest a link between modulation of GJIC and mitogenesis (76). Yamasaki *et al.* (253) report a relationship between increased cell proliferation and

decreased GJIC in a liver model. Chandross *et al.* (221), however, present data concerning the effect of transforming growth factor ß (TGFß) and pituitary extract on GJIC and cell proliferation of Schwann cells, suggesting that factors which stimulate proliferation simultaneously enhance coupling, whereas factors that inhibit proliferation reduce GJIC. Our observations suggest a more complex relationship between modulation of GJIC and cell proliferation, since SMC mitogens may either reduce or enhance GJIC between these cells, depending on the growth factor or cytokine used. Observations in favour of this more complex relationship between GJIC and mitogenesis were previously reported by Madhukar *et al.* (214): epidermal growth factor acted as a mitogen on human keratinocytes while TGFß seemed to suppress DNA synthesis, although both factors inhibited GJIC between these cells. The complex nature of the relationship between modulation of GJIC, cell proliferation and the process of atherosclerosis is further reflected by Rennick *et al.* (67) who observed that gap junctions, measured by means of Cx43 immunolabelling, are numerous between SMC of the synthetic phenotype, a state in which SMC may proliferate. Only a few gap junctions were found in SMC of the contractile phenotype, a state in which cell proliferation does not occur. Recently, Blackburn *et al.* (79) observed that early stages of human atherosclerosis are characterized by increased expression of immunodetectable Cx43 gap junctions in the intima. As the disease progresses, however, the quantity of junctions declines, ultimately to levels below those of the undiseased vessel. Unfortunately, nothing is known yet about the functionality of these immunodetectable gap junction proteins.

In conclusion, the results of our study clearly demonstrate that effects of SMC mitogens on GJIC are not univocal and thus cannot be generalized. Furthermore, the results indicate that it may be useful to separate transient and persistent effects on GJIC with respect to expected or predicted consequences. In atherosclerotic lesions a mixture of growth factors and cytokines will be present. Therefore, it is interesting to examine the interactive effects of combinations of growth modulating factors on GJIC between SMC, in order to mimic the *in vivo* situation more adequately. At the moment, this is further investigated in our laboratory.

Acknowledgements

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Chapter 4
Interactive Effects on Gap Junctional Intercellular Communication by Human Smooth Muscle Cell Mitogens

A. Mensink, T. van der Wijk, I. Struik, L.H.J. de Haan,
A. Brouwer and J.H. Koeman
Submitted

Abstract

Disturbance of smooth muscle cell (SMC) growth control is a key event in the pathogenesis of atherosclerosis. The present study demonstrates that combinations of mitogens exhibit interactive effects on gap junctional intercellular communication (GJIC) between human SMC. GJIC was dramatically reduced upon incubation with tumor necrosis factor α (TNFα) and interferon-γ (IFN-γ). Addition of basic fibroblast growth factor (bFGF) to this combination did not restore GJIC. When cells were exposed to a combination of TNFα and bFGF, control levels of GJIC were obtained which implies an antagonistic interactive effect. Combination of IFN-γ and bFGF resulted in reduced communication at roughly the level of IFN-γ incubations. When platelet-derived growth factor BB (PDGF-BB) was combined with TNFα and/or IFN-γ, the reduction in GJIC upon incubation with these combinations of factors did not differ from the reduction provoked by TNFα and IFN-γ in the absence of PDGF-BB. Superoxide radicals were shown to be involved in the inhibition of GJIC upon incubation with TNFα, but play barely a role in IFN-γ incubations. Immunofluorescence studies revealed that connexin 43 staining was reduced in SMC cultures incubated with a combination of TNFα and IFN-γ. In conclusion, this study demonstrates that interactive effects of mitogens should be taken into consideration when studying growth control mechanisms of SMC.

Introduction

Atherosclerotic lesions ('plaques') are characterized as focal thickenings of the intimal layer in the artery wall. In the course of the disease process, blood monocytes and lymphocytes infiltrate into the intima (3-6). Lesion progression is also accompanied by the indolent proliferation of smooth muscle cells (SMC) and macrophages (8-10), changes in extracellular matrix synthesis and accumulation of intra- and extracellular lipids (7,13).

Disturbance of SMC growth control mechanisms is seen as a key event in the pathogenic process (29,32), in which growth factors and cytokines produced by macrophages, lymphocytes and SMC may play a central role (24,87). Growth factors and cytokines bring about a form of intercellular communication through binding to specific receptors at the cell surfaces of nearby cells. Apart from this receptor-mediated signalling mechanism, direct intercellular communication occurs via gap junctions in plasma membranes. Through these plasma membrane channels ions, metabolites and other small molecules (<1000 D) can be exchanged between cells. Gap junctional intercellular communication (GJIC) is regarded as an important mechanism in the control of cell growth, cell differentiation and tissue homeostasis (69,76). Altered GJIC may affect the pathogenesis of atherosclerosis. Atherogens like oxidized low density lipoprotein (LDL), cigarette smoke condensate, oxysterols and aldehydes reduce GJIC between SMC (57-60). Modulation of GJIC is also likely to play an important role in the process of carcinogenesis; *in vitro* and *in vivo* studies provide evidence for the involvement of GJIC in tumor promotion (71).

Previous work (242,254) demonstrated that SMC mitogens known to be present in atherosclerotic lesions, such as platelet-derived growth factor AA (PDGF-AA), platelet-derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF), interleukin 6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) are able to modulate GJIC between human vascular SMC, suggesting that receptor-mediated signalling and GJIC may be linked. Furthermore, we demonstrated that the action of growth factors and cytokines on GJIC between SMC is mitogen-dependent: incubation with PDGF-AA, PDGF-BB or IL-6 results in a transient reduction in GJIC; exposure to bFGF causes a transient increase in GJIC. IFN-γ and TNFα reduce GJIC for at least 24 h, suggesting that these two cytokines may share some mechanistic similarities with exogenous tumor promoters.

In the SMC micro-environment of the atherosclerotic plaque a mixture of growth factors and cytokines will be present. This may result in interactive effects of these factors on SMC characteristics. The present study focuses on the interactive effects of combinations of mitogens on GJIC between SMC, in order to mimic the *in vivo* plaque

situation more adequately. Possible interactive consequences of these combinations of mitogens on proliferation of SMC were examined as well. In TNFα and IFN-γ incubations, the role of superoxide radicals in the inhibition of GJIC and the immunofluorescence of connexin 43 (Cx43) were studied as well.

Materials and methods

Chemicals

Recombinant human TNFα (molecular weight 36 kD; specific activity 7.69 x 10^7; 1.08 x 10^8 and 1.43 x 10^8 U/mg) and recombinant human IFN-γ (molecular weight 34 kD; specific activity batch-1: 2.5 x 10^7 U/mg; specific activity batch-2: 4.75 x 10^7 U/mg) were obtained from Genzyme Diagnostics (Cambridge, MA). PDGF-BB (molecular weight 30 kD), bFGF (molecular weight 17 kD), minimum essential medium (modified) with Earle's salts (EMEM), fetal bovine serum (FBS), gentamicin, fungizone and 10 x phosphate-buffered saline (PBS; without calcium and magnesium) were supplied by Gibco BRL (Paisley, UK). Superoxide dismutase (SOD; from bovine erythrocytes, specific activity 5000 U/mg) and cell proliferation kits based on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Dulbecco's 'A' PBS was from Oxoid Ltd. (London, UK). Trypsin 1:250 was from Difco (Detroit, MI). Sodium pyruvate was from BDH Chemicals Ltd. (Poole, UK). *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), potassium phosphate, lithium chloride and methanol were purchased from Merck (Darmstadt, Germany). Triton X-100, Lucifer yellow CH and bovine serum albumin (BSA) fraction V were obtained from Sigma Chemical Co. (St. Louis, MO). Monoclonal mouse anti-Cx43 (clone Z039) was obtained from Zymed Laboratories Inc. (San Francisco, CA). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins and Dako Pen were purchased from DAKO A/S (Glostrup, Denmark). Vectashield mounting medium and normal rabbit serum (NRS) were supplied by Vector Laboratories (Burlingame, CA). Slides and coverslips were from Rofa-Mavi (Beverwijk, Netherlands).

Culture of primary human SMC

Primary human SMC were isolated from arteries of human umbilical cords by an explant technique. After careful removal of the adventitial layer, the arterial tissue was cut into small pieces. Explants were incubated in EMEM supplemented with 10% FBS, 50 μg/ml gentamicin and 1.25 μg/ml fungizone in a 37°C, 5% CO_2 humidified atmos-

phere. Cells were allowed to grow for 3-4 weeks until subculturing. Cells displayed the well known 'hills and valleys' pattern after reaching confluence (243). Experiments were performed on cells in passage 2-5.

Experimental procedure

For measurement of GJIC, SMC were grown in 35 mm culture dishes (Greiner B.V., Alphen a/d Rijn, Netherlands) until confluency. Serum containing culture medium was removed whereafter cells were washed with PBS (Oxoid). EMEM supplemented with antibiotics and 0.1 - 0.5 % BSA was added to the cells. Cells were then incubated with the growth modulating factors for the indicated periods of time. Some results in table 4.3 were obtained in incubations in which cells were exposed to IFN-γ for 24 h; during the last hour of this incubation several concentrations of TNFα were added to these cells as well. Just prior to GJIC measurement, HEPES buffer (pH 7.4) was added to the incubations (final concentration 20 mM) to stabilize the pH during microinjection and fluorescence microscopy.

Measurement of GJIC

GJIC was determined after microinjection of a 20% Lucifer yellow CH (in 0.33 M lithium chloride) solution in a single cell (223). In each SMC culture at least 20 individual cells were microinjected using a vertical injection system (Olympus Injectoscope IMT-2-syf) (224) with a dye filled capillary glass tip (Clark, Pangbourne, UK). The capillary glass tip was prepared using an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of 1 μm. The Lucifer yellow CH filled cells were checked with phase-contrast and fluorescence microscopy directly after microinjection. Fifteen to twenty minutes after the first injection the number of communicating cells was determined using fluorescence microscopy. The average number of fluorescent cells was calculated for each incubation. The average number of communicating cells in control incubations was taken as 100% GJIC. Each experiment was performed in duplicate. At least two but predominantly three or more independent tests were done. Data presented are the average values of the performed tests.

Cytokine batch differences in measurement of GJIC

In agreement with our previous work (242), two separate TNFα batches which were generally used in the experiments described in this paper, inhibited GJIC between SMC with $\sim$40% at 0.5 nM (data not shown). However, in a few additional experiments with 0.5 nM of a 'deviant' batch of TNFα, only $\sim$20% inhibition of GJIC was observed (data not shown). The cause for these quantitative differences in effect is not clear. While

performing experiments with bFGF in combination with IFN-γ, we were faced with differences between two IFN-γ batches as well. 1 h incubations with 0.5 nM of either IFN-γ batch alone resulted in a 20% reduced GJIC between SMC. However, when SMC were incubated for 24 h with these IFN-γ batches, dissimilarity occurred. 0.5 nM IFN-γ batch-1 inhibited GJIC still 20%, whereas the same concentration of batch-2 resulted in a much stronger reduction in GJIC of approximately 50% (data not shown). The cause for this discrepancy in effects is not clear. The relatively high SD (13.7%) for 24 h incubations with 0.5 nM IFN-γ batch-2 may indicate that perhaps the 'age' of IFN-γ stock solutions (in PBS/BSA, stored at -80°C) contributes to this phenomenon. At first use, 24 h incubations with IFN-γ batch-2 inhibited GJIC for almost 70%. Via 50% inhibition, its potency to reduce GJIC diminished to 40% within 6 weeks of time. The fact that IFN-γ batch-1 was supplied by the manufacturer as a solution, whereas batch-2 was obtained as a lyophilized powder is in agreement with this hypothesis.

Cell proliferation assay

SMC (2000 - 5000) were plated ($n \geq 16$) in serum containing culture medium in 96 wells microtiter plates (tissue culture grade, Greiner B.V., Alphen a/d Rijn, Netherlands); 100 μl medium per well. After attachment of these cells (for at least 4 h), the culture medium was removed. Cells were then incubated with EMEM supplemented with antibiotics and 0.5% BSA. Growth factors and/or cytokines were added subsequently to the cells (day 1). The media were replaced on day 5 of incubation. Growth factors and/or cytokines were added to culture medium containing antibiotics and 2% FBS, whereafter cells were exposed to these factors for three more days. On day 8, SMC were incubated with the MTT solution and solubilization buffer, according to the instructions of the manufacturer. On day 9, the formazan dye formed in these cells was quantified by reading the absorbance at 595 nm, using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

Immunofluorescence assay

SMC were cultured on glass slides until these cells made substantial (membrane) contacts with neighbouring cells. Then, cells were exposed for 24 h to EMEM with antibiotics and 0.5% BSA (control) or to this medium completed with 0.5 nM TNFα and/or 0.5 nM IFN-γ. After these incubations, cells were washed twice with PBS (Oxoid). Then, cells were fixed for 5 min in ice-cold methanol. Cells were dried at room temperature and stored at 2-8 °C. Fixed cells were subsequently put in PBS for 5 min, followed by treatment with 0.2% Triton X-100 in PBS for 1 h. From these two incubations onward, PBS without calcium and magnesium (Gibco) was used. Then, the cells

were rinsed in PBS and incubated with 2% BSA in PBS for 30 min. After washing in PBS, the cells were incubated overnight with the monoclonal anti-Cx43 antibody (1:1000 in 10% NRS in PBS) at room temperature. Subsequent washing in PBS (3 changes of 5 min), incubation with 2% BSA in PBS (30 min) and another washing step in PBS preceded a 3 h incubation with the polyclonal FITC-conjugated rabbit-anti-mouse antibodies (1:20 in 10% NRS in PBS) at room temperature. After washing in PBS (3 changes of 5 min), cells were enclosed in vectashield and coverslips were applied. Slides were stored at 2-8 °C until examination with a Nikon optiphot-2 with photo-attachment, equipped with appropriate filters for epifluorescence. Photographs were taken using a Kodak 3200 asa tmz film. Exposure time was ± 25 sec. Quantification of fluorescence was performed on scanned negatives (Horizon scanner, Agfa, Germany; 1000 dpi, 1350 x 870 pixels). Fluorescent objects were defined as a minimum of 4 pixels (programme NIH 1.6.0). Thus, 1 - 3 pixels were regarded as noise to correct for the granularity of the film. Tresholds were 155 (gray) - 255 (black).

Cytotoxicity assay

SMC were grown in six-well tissue culture plates (Costar Europe Ltd., Badhoevedorp, Netherlands). When confluent, cells were incubated as for GJIC measurement. After treatment, media were taken from the wells and were centrifuged. Cells were scraped from the bottom of the wells after addition of 0.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.5, followed by a sonification step in ice water (5 min, Sonorex RK 100 (Bandelin GmbH, Berlin, Germany)) and a centrifugation step. Lactate dehydrogenase (LDH) activity was measured in the supernatants (225) and LDH leakage was calculated. Each experiment was performed in duplicate and at least two independent tests were done.

Statistics

Statistical analyses of the data were performed using Students *t*-test ($P < 0.05$).

Results

Smooth muscle cell GJIC

Primary human SMC from umbilical cord arteries display a distinct GJIC (242,254). In the experiments described here, Lucifer yellow spread over an average of 38 - 95 cells in control incubations. Differences in the number of communicating SMC in separate experiments may result from SMC heterogeneity between cultures and may also

depend on SMC density at confluence.

With the exception of the experiments concerning the combination of IFN-γ and bFGF, SD for duplicate incubations in separate experiments generally did not exceed 10% and were often much less.

Combination of TNFα and bFGF

Addition of 0.5 nM TNFα in combination with 0.5 or 2.0 nM bFGF to human SMC for 24 h gave rise to GJIC values that did not differ from control values (table 4.1). The GJIC values of the combined exposures differed significantly from those obtained upon incubation with 0.5 nM TNFα or 0.5/2.0 nM bFGF alone for 24 h. Thus, at 24 h, effects of TNFα and bFGF on GJIC seem to counteract each other. Upon 30 h incubation of SMC with the combination of 0.5 nM TNFα and 2.0 nM bFGF, GJIC was significantly reduced as compared to control values (table 4.1). This reduction of ~34% corresponds well with the reduction caused by incubation with 0.5 nM TNFα alone, indicating that bFGF at 30 h of incubation apparently does not affect GJIC anymore.

Table 4.1

GJIC (% of control) upon incubation of human SMC with TNFα and bFGF

exposure	time (h)	GJIC (%)
0.5 nM TNFα	24	65.0 ± 2.1 a
0.5 nM bFGF	24	122.6 ± 2.6 a
2.0 nM bFGF	24	139.0 ± 3.7 a
0.5 nM TNFα + 0.5 nM bFGF	24	98.5 ± 5.8
0.5 nM TNFα + 2.0 nM bFGF	24	109.0 ± 5.0
0.5 nM TNFα + 2.0 nM bFGF	30	66.5 ± 1.1 a,b

Average SD for control incubations was 2.8%. GJIC values from incubations with TNFα and bFGF alone were reproduced from our previous reports (242,254). a: significantly different from control incubations, b: significantly different from incubations with 0.5 nM TNFα + 2.0 nM bFGF for 24 h.

Combination of IFN-γ and bFGF

The interpretation of the interactive effects of IFN-γ and bFGF on GJIC between SMC is difficult, due to the IFN-γ batch differences (see materials and methods section).

The combination of 0.5 nM IFN-γ batch-1 with 0.5 nM bFGF for 24 h resulted in a significant reduction in GJIC of 38% as compared to control values (table 4.2), which was significantly different from incubations with IFN-γ batch-1 or bFGF alone. However, this 38% reduction in GJIC does not differ significantly from the 32% reduction in GJIC which was observed in incubations with 0.5 nM IFN-γ batch-1 in combination with 2.0 nM bFGF. When compared to incubations with IFN-γ batch-1 alone, the combination of 0.5 nM bFGF and 0.5 nM IFN-γ batch-1 significantly further decreased GJIC, whereas the combination of 2.0 nM bFGF and 0.5 nM IFN-γ batch-1 did not. Experiments with bFGF in combination with IFN-γ batch-2 gave rise to somewhat different results. Upon combination of these factors, GJIC decreased to the level of inhibition observed in experiments with IFN-γ batch-2 alone. However, a non-significant increase in GJIC was observed when combinations of IFN-γ batch-2 and 2.0 nM bFGF were compared with combinations of IFN-γ batch-2 and 0.5 nM bFGF (table 4.2). Although it is difficult to interpret these varying results, the overall effect of combinations of IFN-γ with bFGF differed from the effect of TNFα and bFGF combinations. TNFα and bFGF were able to counteract each others effect, while in IFN-γ and bFGF combinations this did not seem to occur consistently.

Table 4.2
GJIC (% of control) upon 24 h incubation of human SMC with IFN-γ and bFGF

	0 nM bFGF	0.5 nM bFGF	2.0 nM bFGF
0 nM IFN-γ	100	122.6 ± 2.6 a,b	139.0 ± 3.7 a.b
0.5 nM IFN-γ batch-1	80.6 ± 2.3 a,c	61.9 ± 5.2 a,b,c	68.3 ± 11.9 a,c
0.5 nM IFN-γ batch-2	48.0 ± 13.7 a,c	51.4 ± 5.9 a,c	69.5 ± 17.2 a,c
0.5 nM IFN-γ batch-1/2		57.7 ± 7.5 a,c	69.1 ± 14.9 a,c

Average SD for control incubations was 13.6%. GJIC values from incubations with IFN-γ and bFGF alone were reproduced from our previous report (254). a: significantly different from control incubations, b: significantly different from IFN-γ incubations, c: significantly different from bFGF incubations.

Combination of TNFα and IFN-γ

Addition of 0.5 nM IFN-γ for 24 h, combined with 1 h additions of several concentrations of TNFα resulted in significant reductions in GJIC between SMC (table 4.3). At 0.5 nM for both factors, GJIC was reduced to 43% as compared to control values. When the IFN-γ concentration increased to 1.5 nM under the same experimental conditions, no extra inhibition of GJIC was observed. A similar, ~57% reduction in GJIC between SMC was also established when these cells were exposed to the combination of 0.5 nM IFN-γ and 0.5 nM TNFα for only 1 h. Upon simultaneous addition of 0.5 nM TNFα and 0.5 nM IFN-γ for 24 h, GJIC between human SMC was vigorously reduced. Only 14% GJIC was left between these cells, which was significantly different from control values and from exposures to TNFα, IFN-γ batch-1 or -2 alone (table 4.3). In series of additional experiments with a 'deviant' batch of TNFα (see materials and methods section), the simultaneous exposure of SMC to 0.5 nM TNFα and 0.5 nM IFN-γ batch-2 for 24 h resulted in a GJIC of 30.5 ± 2.6%. Although this ~70% reduction in GJIC is still considerable, the reduced capacity of this batch of TNFα concerning GJIC inhibition is clearly expressed even in combination with IFN-γ. Overall, the effects of IFN-γ in combination with TNFα on reduction of GJIC may be described as arithmetically additive, since the effect of the combination (at 0.5 nM ~57% inhibition) is similar to the sum of both individual effects (at 0.5 nM ~40% and ~20% inhibition for TNFα and IFN-γ batch-1 respectively). In addition, when TNFα and IFN-γ were combined for 24 h, the strong reduction (~86%) in GJIC resembled additive (compared to IFN-γ batch-2) or even synergistic (compared to IFN-γ batch-1) effects.

To determine whether the strong reduction in GJIC upon 24 h incubation with 0.5 nM TNFα and 0.5 nM IFN-γ was (partly) due to loss of membrane integrity, LDH activity was measured in culture media and cell homogenates. No membrane cytotoxicity was observed upon 24 h of incubation with a combination of these cytokines. Values of LDH activity in cell homogenates after exposure varied between 93 and 101% of control values (average value 98%, data not shown). These cytokines may however have influenced the viability of SMC in another way. It was previously reported (118) that the combination of TNFα and IFN-γ may induce apoptosis in (subpopulations of) cultured human SMC. To exclude a possible dominating influence of apoptosis on the strong inhibition of GJIC upon incubation with TNFα and IFN-γ together, 'restoration' experiments were carried out. SMC were incubated with 0.5 nM TNFα and 0.5 nM IFN-γ for 24 h. Then, cells were washed with PBS and incubated with control (BSA) medium for another 24 h, whereafter GJIC was measured. These SMC communicated at the same level as control cells; that is 100.1 ± 2.8%. Thus, apoptosis does not underlie the observed strong reduction of GJIC upon exposure to these cytokines as well. In addition,

no morphological changes between control cells and exposed cells were observed in the great majority of the experiments performed with IFN-γ in combination with TNFα. In a couple of experiments however, a few cells could have been apoptotic: they shrank and retracted from their neighbour cells. In the direct area around these cells, no microinjection of Lucifer yellow was performed.

Combination of TNFα, IFN-γ and bFGF

When human SMC were exposed to combinations of TNFα, IFN-γ and bFGF for 24 h, GJIC was again strongly reduced to a level comparable to 24 h incubations with TNFα and IFN-γ (80 - 86% inhibition, table 4.3). Thus, addition of bFGF to the combination of TNFα and IFN-γ did not restore GJIC even a bit. In this combination of three cytokines, effects on GJIC are not simply (arithmetically) additive, since an extra effect of bFGF, compared to incubations of IFN-γ together with TNFα, could not be observed. No membrane cytotoxicity was observed when cells were incubated with the combination of 0.5 nM TNFα, 0.5 nM IFN-γ and 2.0 nM bFGF: LDH activity in cell homogenates varied between 97 and 99% of control values (data not shown).

Combination of PDGF-BB and TNFα and/or IFN-γ

One hour incubations with 0.5 nM PDGF-BB reduced GJIC between SMC significantly to 72.9 ± 5.5%, as compared to control cells. When 0.5 nM PDGF-BB was combined with 0.5 nM TNFα for 1 h, GJIC was reduced to ~63% as compared to control incubations (Fig. 4.1); a value similar to the decrease in GJIC caused by 0.5 nM TNFα alone. The combination of 0.5 nM PDGF-BB and 0.5 nM IFN-γ for 1 h reduced GJIC values between these cells with ~20% of control values (Fig. 4.1). This reduction in GJIC is comparable with the inhibition of GJIC caused by IFN-γ alone. Approximately 55% reduction in GJIC was obtained when SMC were incubated with the combination of 0.5 nM PDGF-BB, 0.5 nM TNFα and 0.5 nM IFN-γ for 1 h (Fig. 4.1). This decrease in GJIC is significantly different from GJIC inhibition as a result of TNFα, IFN-γ or PDGF-BB incubations alone, but does not differ from the reduction in GJIC caused by the combination of 0.5 nM TNFα and 0.5 nM IFN-γ for 1 h (see also table 4.3). Thus, effects of PDGF-BB in combination with TNFα and/or IFN-γ are not arithmetically additive. However, it cannot be concluded yet that antagonism underlies the observed effects, since TNFα and IFN-γ were used in concentrations that inhibited GJIC maximal when administered alone (242,254). As a consequence, intracellular processes involved may already have functioned at their maximum.

Table 4.3
GJIC (% of control) upon incubation of human SMC with TNFα, IFN-γ and bFGF

exposure	time (h)	GJIC (%)
0.1 nM TNFα	1	69.5 ± 5.2 a
0.5 nM TNFα	1	60.5 ± 2.6 a
1.0 nM TNFα	1	60.5 ± 5.7 a
0.5 nM IFN-γ	1	81.5 ± 2.4 a
0.5 nM TNFα	24	65.0 ± 2.1 a
0.5 nM IFN-γ	24	80.6 ± 2.3 a
0.5 nM bFGF	24	122.6 ± 2.6 a
2.0 nM bFGF	24	139.0 ± 3.7 a
0.5 nM IFN-γ/0.1 nM TNFα	24/1	57.5 ± 6.8 a,b,c
0.5 nM IFN-γ/0.5 nM TNFα	24/1	43.4 ± 2.6 a,b,c
0.5 nM IFN-γ/1.0 nM TNFα	24/1	43.4 ± 4.0 a,b,c
1.5 nM IFN-γ/0.5 nM TNFα	24/1	43.6 ± 4.9 a,b,c
0.5 nM IFN-γ/0.5 nM TNFα	1	43.3 ± 3.1 a,b,c
0.5 nM IFN-γ/0.5 nM TNFα	24	14.4 ± 3.6 a.b.c
0.5 nM IFN-γ/0.5 nM TNFα/0.5 nM bFGF	24	20.6 ± 9.3 a,b,c,d,e,f
0.5 nM IFN-γ/0.5 nM TNFα/2.0 nM bFGF	24	14.5 ± 8.9 a,b,c,d,e,f

Average SD for control incubations was 2.5 - 10.2%. (Estimates of) GJIC values from incubations with TNFα, IFN-γ and bFGF alone were reproduced from our previous reports (242,254). IFN-γ batch-1 was used in these experiments, except for the 24 h incubations of IFN-γ in combination with TNFα in which both IFN-γ batch-1 and IFN-γ batch-2 were used. a: significantly different from control incubations, b: significantly different from TNFα incubations, c: significantly different from IFN-γ incubations, d: significantly different from bFGF incubations, e: significantly different from 24 h incubations with TNFα + bFGF, f: significantly different from 24 h incubations with IFN-γ + bFGF.

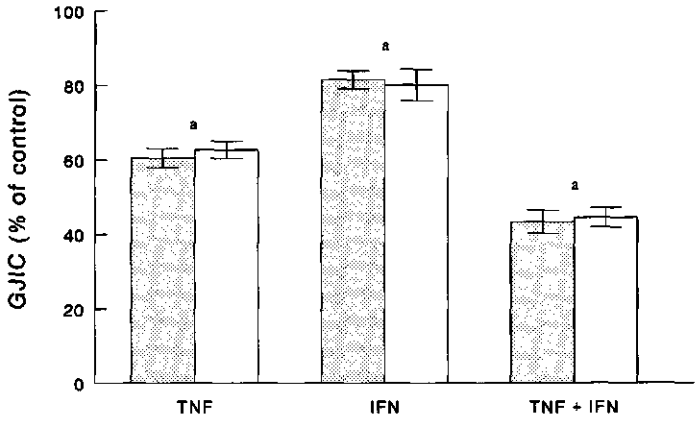

Fig. 4.1. GJIC (% of control) upon 1 h incubations of human SMC with PDGF-BB, TNFα and IFN-γ. Dotted (grey) bars represent incubations with 0.5 nM TNFα and/or 0.5 nM IFN-γ, which are partly reproduced from our previous reports (242,254). Open bars represent these incubations of TNFα and/or IFN-γ in combination with 0.5 nM PDGF-BB. Average SD for control incubations was 2.2%. IFN-γ batch-1 was used in these experiments. a: significantly different from control incubations.

Incubations with SOD

In a preceding paper (242) we reported that free radical production by SMC might play a role in the observed reduction in GJIC caused by TNFα, since pretreatment of these cells with ascorbic acid, α-tocopherol or glutathione (GSH) prevented this inhibition of GJIC. In order to study whether this radical based inhibition of GJIC is a unique feature for TNFα and to gain more insight in the radical species involved, we studied the effect of SOD on TNFα and/or IFN-γ induced reduction of GJIC.

When administered alone, SOD did not affect GJIC (table 4.4). Addition of 200 U/ml SOD simultaneously with TNFα for 1 h prevented the inhibition of GJIC between SMC which occurs upon incubation with TNFα alone (table 4.4). When SMC were pretreated with 0.5 nM TNFα for 1 h and 200 U/ml SOD was added subsequently to this TNFα incubation for another hour, GJIC returned to control values: 96.8 ± 2.5% (data not shown). This amount of SOD was still able to shift GJIC values back to control levels when SMC were incubated with 0.5 nM TNFα for 23 h prior to the 1 h addition of SOD (table 4.4). Thus, the superoxide radical seems to be involved in GJIC reduction by TNFα, since incubation with SOD, even hours after the addition of TNFα, restored GJIC

to control values. The effect of SOD on IFN-γ incubations was more complex. Upon 1 h incubation with IFN-γ, 200 U/ml SOD significantly elevated GJIC values with ~10% as compared with IFN-γ GJIC values (table 4.4). However, 1 h exposures of SOD could not (partly) restore the inhibition of GJIC between SMC in 24 h incubations with IFN-γ. The combination of 0.5 nM TNFα, 0.5 nM IFN-γ and 200 U/ml SOD for 1 h gave rise to a GJIC value that corresponds with the value obtained in incubations with IFN-γ and SOD for 1 h: 89.0 ± 4.6% (data not shown). Finally, when 24 h incubations of 0.5 nM TNFα together with 0.5 nM IFN-γ were completed with an one hour incubation of as much as 1000 U/ml SOD, the antioxidant enzyme could not even partly neutralize the strong inhibition of GJIC caused by these cytokines (data not shown).

Table 4.4

GJIC (% of control) upon incubation of human SMC with TNFα or IFN-γ and SOD

time (h)	exposure	GJIC (%) - SOD	GJIC (%) +SOD
1		100	99.0 ± 5.3 b,c
1	TNFα	60.5 ± 2.6 a	98.2 ± 6.4 b
24	TNFα	82.0 ± 3.1 a	96.2 ± 3.1 b
1	IFN-γ	81.5 ± 2.4 a	91.4 ± 4.8 a,c
24	IFN-γ	80.6 ± 2.3 a	75.1 ± 4.3 a

SOD was added during the last hour of the incubations at 200 U/ml. Concentrations of TNFα and IFN-γ were 0.5 nM. Average SD for control incubations in various sets of experiments did not exceed 5.6%. GJIC values from incubations with TNFα and IFN-γ alone were reproduced from our previous reports (242,254), except for 24 h incubations with TNFα since the 'deviant' batch of TNFα (see materials and methods section) was used in these 24 h experiments. Both IFN-γ batch-1 and IFN-γ batch-2 were used in these experiments. a: significantly different from control incubations, b: significantly different from TNFα incubations, c: significantly different from IFN-γ incubations.

Immunofluorescence studies

Incubation of SMC with 0.5 nM TNFα in combination with 0.5 nM IFN-γ for 24 h resulted in a reduced Cx43 immunostaining with monoclonal anti-Cx43 as compared with untreated cells. In Fig. 4.2 the results of three independent experiments are shown. In 'valley' regions of control SMC (Fig. 4.2 A,C,E) the fluorescent Cx43 spots are

frequent. Upon incubation with the combination of TNFα and IFN-γ, the amount of fluorescent spots decreased in valley regions, as can be seen in Fig. 4.2 (B,D,F). However, in the so called 'hill' regions where SMC display a crowded multilayer growth pattern, Cx43 fluorescence was strongly present in both control and cytokine incubations (data not shown). The number of fluorescent objects in valley regions of Fig. 4.2 were quantified (table 4.5). The difference between control cells and exposed cells was evident, although the reduction in fluorescence differed per experiment. Exposure of SMC with 0.5 nM TNFα for 24 h did not seem to affect Cx43 staining compared to control incubations (data not shown). Cx43 staining upon incubation of SMC with 0.5 nM IFN-γ for 24 h did not give simple univocal results (data not shown).

SMC proliferation

The individual factors TNFα, IFN-γ, bFGF and PDGF-BB all stimulated SMC proliferation. In table 4.6 absorbance values (as indicator of cell proliferation) are given for the incubations of SMC with these mitogens. Furthermore, several combinations of mitogens were tested for growth promoting activity as well. The results (table 4.6) show that all combinations tested stimulated SMC proliferation significantly in comparison with control cells. Because of the absence of dose-response curves for the individual factors and inter-experimental variance however, it is not possible to make a statement with regard to possible interactive effects of these factors on cell proliferation. This is clearly demonstrated in incubations with the combinations of TNFα and IFN-γ or TNFα and PDGF-BB. Exposure to the combination of TNFα and bFGF gave rise to somewhat more univocal results. These incubations may result in decreased SMC proliferation as compared to incubation with bFGF alone. Regarding the combination of IFN-γ and bFGF, we noticed that in four incubations, SMC proliferation was more or less equal or reduced compared with cell proliferation as a result of incubation with IFN-γ or bFGF alone. In two incubations however, SMC proliferation was somewhat increased compared to the incubations with the individual factors. The combination of PDGF-BB and IFN-γ seemed to induce SMC proliferation more or less comparable with the induction by IFN-γ alone.

Fig. 4.2. Cx43 immunofluorescence in valley regions of human SMC cultures. Three independent tests were done. Photographs **A, C and E** represent control incubations; photographs **B, D and F** represent the corresponding incubations with 0.5 nM TNFα in combination with 0.5 nM IFN-γ. Bar = 31 μm

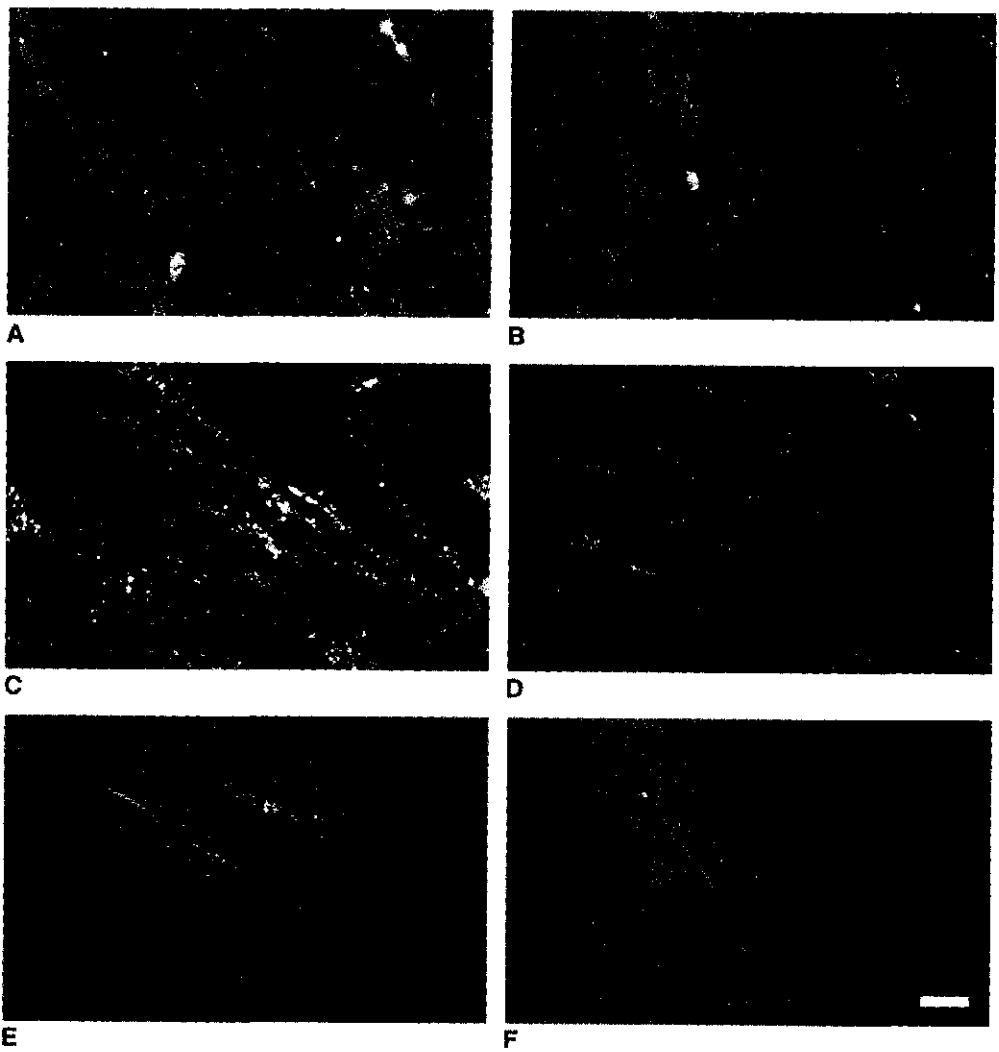
A
B
C
D
E
F

Table 4.5

Quantification of fluorescent objects (arbitrary units) in valley regions of human SMC as photographed in figure 4.2

control	TNFα/IFN-γ	decrease
A: 1956	B: 1200	36%
C: 834	D: 343	59%
E: 302	F: 47	84%

Cells were incubated with control (BSA) medium or with 0.5 nM TNFα in combination with 0.5 nM IFN-γ for 24 h.

Table 4.6

Absorbance values (% of control) of SMC incubations with growth factors and cytokines as measured in the MTT test.

exposure	test 1	test 2	test 3	test 4
control	100 ± 8	100 ± 6	100 ± 7	100 ± 8
0.5 nM TNFα	112 ± 5	113 ± 7	117 ± 6	107 ± 10
0.5 nM IFNγ	123 ± 8	120 ± 5	123 ± 5	126 ± 7
0.5 nM bFGF	129 ± 5	134 ± 5	113 ± 5	
2.0 nM bFGF	129 ± 6	133 ± 6	113 ± 5	
0.5 nM PDGF-BB	117 ± 6	127 ± 4	110 ± 7	
0.5 nM TNFα + 0.5 nM IFNγ	134 ± 11 a,b	125 ± 7 a,b	109 ± 8 a,b	163 ± 9 a,b
0.5 nM TNFα + 0.5 nM bFGF	123 ± 5 a,c	121 ± 6 a,c	115 ± 7	
0.5 nM TNFα + 2.0 nM bFGF	115 ± 6 c	122 ± 6 a,c	115 ± 8	
0.5 nM IFNγ + 0.5 nM bFGF	136 ± 5 b,c	131 ± 8 b	128 ± 8 b,c	
0.5 nM IFNγ + 2.0 nM bFGF	124 ± 5 c	129 ± 5 b,c	124 ± 8 c	
0.5 nM TNFα + 0.5 nM PDGF-BB	127 ± 5 a,d	123 ± 7 a,d	116 ± 9 d	
0.5 nM IFNγ + 0.5 nM PDGF-BB	124 ± 5 d	118 ± 7 d	118 ± 9 b,d	

In all four experiments: n ≥ 16. All growth factor- and cytokine incubations were significantly different from control incubations. a: significantly different from TNFα incubation; b: significantly different from IFNγ incubation; c: significantly different from bFGF incubation; d: significantly different from PDGF-BB incubation.

Discussion

The present study demonstrates for the first time that combinations of growth factors and cytokines exhibit interactive effects on GJIC between human SMC. These interactive effects may be described as either antagonistic, additive or even synergistic, depending on the combination of factors tested. When TNFα was combined with IFN-γ, GJIC between SMC was strongly reduced with 57% (1 h) or 86% (24 h). Addition of bFGF for 24 h to this combination of TNFα and IFN-γ did not restore GJIC even a bit. A consequence of this 80 - 86% inhibition of GJIC was that in some experiments a number of SMC did not communicate with neighbouring cells at all. That is, only the injected cell itself was fluorescent upon examination. This may result in an escape from growth control mechanisms which, in turn, may lead to disturbance of SMC proliferation, a key event in atherosclerosis. When TNFα and bFGF were combined for 24 h, they counteracted each others effects on GJIC to values corresponding with control communication. The mechanism of this TNFα-bFGF interaction on GJIC may probably be best described as antagonism. In contrast to the combination of TNFα with bFGF, no major counteracting effects were observed on GJIC inhibition by combinations of IFN-γ and bFGF for 24 h. Finally, addition of PDGF-BB to TNFα and/or IFN-γ for 1 h did not influence the reduction in GJIC which was obtained by TNFα and/or IFN-γ as such. More research is needed to evaluate these growth factor and cytokine interactions properly. SMC should be exposed to mixtures of non-saturating concentrations of individual factors, in order to obtain dose-response data that would allow more insight about the nature of interactions and the mechanisms involved.

Studies with SOD demonstrated that the superoxide radical may be involved in GJIC reduction by TNFα, since incubation with SOD, even hours after the addition of TNFα, restored GJIC to control values. This observation is consistent with our previous work, in which we demonstrated that pretreatment of SMC with ascorbic acid, α-tocopherol or GSH prevented the TNFα-induced inhibition of GJIC (242). Experiments with SOD previously revealed that superoxide radicals may be involved in GJIC inhibition upon incubation with exogenous chemicals, like phenobarbital, as well (233,255). The apparent production of superoxide radicals by SMC in our experiments is in agreement with the generation of these radicals by human fibroblasts upon exposure to TNFα (256). Furthermore, SOD partly restored IFN-γ effects on GJIC in the short- but not in the long term. When SMC were incubated with TNFα and IFN-γ simultaneously for 24 h, high levels of SOD could not even partly counteract the strong inhibition of GJIC caused by these cytokines. Thus, other mechanisms may affect GJIC more predominantly in long term incubations with TNFα and IFN-γ.

One such mechanism may be represented by the reduced Cx43 staining which was observed in immunofluorescence studies on SMC cultures incubated with TNFα and IFN-γ for 24 h. Further research has to establish whether this reduced immunostaining results from reduced Cx43 expression, or if Cx43 epitope presentation is affected by functional modifications or protein-Cx43 interactions. The difference in Cx43 fluorescence between control cells and cells exposed to TNFα and IFN-γ was evident, although the reduction in fluorescence differed per experiment. These differences may be due to the heterogeneity between these cultures of primary human SMC (254). Alternatively, these differences may originate from differences in the ratio between the amount of cytokines and SMC. This ratio may explain the Cx43 fluorescence in exposed hill regions as well.

TNFα, bFGF, IFN-γ and PDGF-BB all stimulated SMC proliferation, as individual factors as well as in combinations. Unfortunately, not much can be said regarding the nature of interactive effects on cell proliferation, since a considerable inter-experimental variance was observed. In general, SMC proliferation was not extra induced by the addition of a second mitogen. Although comparison of GJIC and cell proliferation is faced with some difficulties, for instance the different time scales of the two types of experiments, the present results indicate that the relationship between GJIC and cell proliferation may be complex as we stated before (254).

Variations in SMC responses, concerning for instance the absolute number of communicating SMC in control incubations, the reduction of Cx43 fluorescence upon incubation with TNFα and IFN-γ and the differences in cell proliferation may represent matters inherent to SMC culturing. Differences in SMC 'batches' (254) or SMC phenotypes (257) and differences in cell densities (because plating efficiences and growth rates cannot be accurately predicted (108)) may underlie these variations.

We can only speculate about the intracellular mechanisms of the described interactive effects. We restricted our reflections to the combinations of TNFα/bFGF, TNFα/IFN-γ and TNFα/IFN-γ/bFGF, since marked effects were observed in these incubations. When TNFα was combined with bFGF, GJIC seemed to be antagonistically influenced by both factors. The 'overall' result of the simultaneous exposure to TNFα and bFGF may be a simple addition of two unrelated mechanisms. When bFGF exposure, for instance, elevates Cx43 levels in SMC analogous to its action in endothelial cells (207), more gap junctions may be formed which may elevate GJIC. However, due to the action of TNFα, a substantial part of these 'extra' channels may be closed. Several other phenomena concerning TNFα-bFGF interactions have been reported. TNFα is likely to increase the number of heparin-binding (fibroblast) growth factor receptors on human SMC (102) and may therefore influence the binding of bFGF on SMC. Hydrogen

peroxide, which may be formed out of superoxide radicals upon incubation with TNFα or which may be produced upon bFGF induction of SMC (176), has been demonstrated to strongly increase the affinity of bFGF for its receptor at the cell surface of SMC (258). Furthermore, TNFα is able to induce 2'-5'oligoadenylate synthethase in SMC (98). This may lead to degradation of RNA and reduced protein synthesis (129), which in turn may affect Cx43 expression. Concerning SMC proliferation, our results may correspond to those of others who observed antagonism between TNFα and bFGF on endothelial cell growth (109). However, TNFα alone inhibited endothelial cell growth in these studies, unlike the stimulation of SMC proliferation seen in our study.

A lot of information is available concerning interactive effects of IFN-γ and TNFα. Synergistic effects of both cytokines were observed studying cytotoxicity/growth inhibition of tumor cells (259-261), induction of oxidative stress, inhibition of energy metabolism and GSH depletion in hepatocytes (262) and hydrogen peroxide production and NO_2 release from macrophages (263). Antagonism was observed in studies concerning proliferation of human fibroblasts (264) and expression of HLA proteins on fibroblast-like synoviocytes (265). In studies with human and rat SMC, synergistic effects of TNFα and IFN-γ were reported concerning apoptosis (118), induction of HLA expression (133), induction of intercellular adhesion molecule-1 (115) and stimulation of NO production (119,120,266). Our observation that combining TNFα and IFN-γ results in additive or synergistic inhibition of SMC GJIC adds a new kind of interactive effect to these lists. Our variable results concerning cell proliferation upon combining TNFα and IFN-γ are not entirely surprising. Stimulating as well as inhibiting effects on SMC proliferation have been described for both individual factors (103-109,126-129,254,258). Concerning their combination, inconsistent reports also exist (103,108,110,134).

Receptor modulation may contribute to the interactive actions of TNFα and IFN-γ (265,267-270). However, actions at the post-receptor level may be of importance as well (271-274). The additive effect on GJIC seen in our 24 h incubation of SMC with IFN-γ combined with 1 h of TNFα is not likely to be due to induction of TNFα receptors, since combinations with 1.0 nM TNFα did not result in a stronger GJIC inhibition than combinations with 0.5 nM TNFα. Furthermore, the same amount of GJIC inhibition was achieved when SMC were incubated with both cytokines for only 1 h, a time span in which complete receptor synthesis is not likely to occur. It cannot be excluded, however, that TNFα induced IFN-γ receptors, since GJIC was synergistically inhibited in 24 h incubations with TNFα and IFN-γ batch-1. The arithmetically additive effects on GJIC occurred despite the fact that both factors bring about their maximal individual effects at 0.5 nM (242,254). This may suggest that IFN-γ and TNFα exhibit (at least in part) their

effect on GJIC through different intracellular pathways. Differences in results in TNFα and IFN-γ incubations in experiments with SOD and differences in GJIC upon IFN-γ/bFGF versus TNFα/ bFGF combinations are also in favour of this suggestion.

Addition of bFGF to the combination of IFN-γ and TNFα for 24 h did not restore GJIC even a bit. Several aspects may be considered in this perspective. Like TNFα, IFN-γ is able to induce 2'-5'-oligoadenylate synthethase activity, which may affect Cx43 expression (129). Furthermore, it was demonstrated that effects of bFGF on GJIC varied. bFGF enhanced GJIC in endothelial cells and cardiac fibroblasts (207-208), probably by inducing Cx43 expression. However, bFGF reduced GJIC in cardiac myocytes (209). It was suggested that differences in intracellular milieu affect bFGF actions (209). Cytokines like TNFα and IFN-γ may affect this intracellular milieu and may therefore influence bFGF actions. Cytokine-induced differences in intracellular milieu may underlie the observed differences in interactive effects on GJIC between TNFα/bFGF and IFN-γ/bFGF combinations as well.

In conclusion, interactive effects should be taken into consideration when studying disturbance of growth control mechanisms, since multiple growth factors and cytokines may simultaneously be present in tissues. The factors tested in this study have all been shown to be present in human atherosclerotic lesions (4,94,142,194). It is not unlikely that circumstances vary considerably in plaques. The exact composition of the mixture of growth factors and cytokines may differ locally, for instance due to the presence of many or just a few macrophages, foam cells and T-lymphocytes. In order to gain more insight in the pathological process of atherosclerosis, further research should be directed to the presence of growth factor- and cytokine receptors on SMC in lesions and to the interactive effects of factors on SMC characteristics.

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Chapter 5
Macrophages Influence Gap Junctional Intercellular Communication between Smooth Muscle Cells in a Co-Culture Model

A. Mensink, F.P.J. Mul, T. van der Wijk, A. Brouwer and J.H. Koeman
Submitted

Abstract

The present study demonstrates for the first time that cells cultured on pore membrane inserts (macrophages) modulate gap junctional intercellular communication (GJIC) between a second cell type (smooth muscle cells (SMC)) co-cultured in Transwell-COL cell culture chambers. Unstimulated J774A.1 murine macrophages reduced GJIC between human SMC. Stimulation of J774A.1 cells by lipopolysaccharide (LPS) or interferon-γ abrogated this modulation of GJIC. Unstimulated human monocyte-macrophages did not affect GJIC between human SMC. Upon stimulation of these monocyte-macrophages with LPS, a substantial increase in GJIC between co-cultured SMC was observed. Thus, activation of macrophages alters their interaction with co-cultured SMC. Since these results were obtained in an indirect co-culture system in which direct cell-cell contact is prevented, it is hypothesized that soluble factors released by macrophages may be involved in this modulation of GJIC between SMC. The possible nature of the responsible soluble factors is discussed in the context of atherosclerosis.

Introduction

Atherosclerotic plaques are heterogenous with respect to cellular composition: smooth muscle cells (SMC), macrophages and T lymphocytes have all been demonstrated to be present in human atherosclerotic lesions (5,275). Disturbance of SMC growth control mechanisms is seen as a key event in the pathogenesis of atherosclerosis (29,32). Interactions between SMC and blood-borne cells may therefore be important in the etiology of the disease process. Macrophages, for instance, may influence SMC through modulation of lipoproteins, secretion of growth factors and cytokines and release of enzymes and reactive oxygen species (276).

In most studies, macrophage-derived factors have been added as purified components or as conditioned media to SMC cultures. These kinds of experiments have several disadvantages. In testing one single factor, the complexity of the macrophage secretion pattern is not taken into account at all. Upon using conditioned media, unstable factors may be lost during media collection or processing.

To overcome these disadvantages, SMC and macrophages have been co-cultured either directly (cell - cell contact) or indirectly. In indirect co-culture systems no cell - cell contact occurs, since SMC and macrophages are separated by, e.g. pore membranes. Through these pore membranes diffusion of soluble mediators may occur. In indirect co-culture experiments it was demonstrated that macrophages influenced SMC phenotype and stimulated SMC proliferation (277,278). In contrast with these results, Fan *et al.* (279,280) reported that macrophages inhibited SMC proliferation in indirect co-culture experiments. Zhang *et al.* (281) demonstrated that both soluble factors and direct macrophage - SMC cell-cell contact may influence SMC characteristics.

Previous work (242,254,chapter 4) demonstrated that macrophage- and lymphocyte-derived growth factors and cytokines were able to modulate gap junctional intercellular communication (GJIC) between SMC in an interactive fashion. GJIC is regarded as an important mechanism in the control of cell growth, cell differentiation, and tissue homeostasis (69,70). Therefore, modulations of GJIC may have pathophysiological consequences. *In vitro* and *in vivo* studies provide evidence that inhibition of GJIC plays a role in the promotion phase of carcinogenesis (71,72,74,77). Altered GJIC may affect the pathogenesis of atherosclerosis as well. Atherogens like oxidized low density lipoprotein (LDL), cigarette smoke condensate, oxysterols and aldehydes reduce GJIC between SMC (57-60).

The present study was aimed at studying the effect of macrophages on GJIC between SMC in an indirect co-culture system. Macrophages were cultured on 24.5 mm pore membrane inserts which were placed inside six-well cluster plates containing human

SMC on glass coverslips. After co-culturing, the hetero-cellular influence of macrophages on GJIC between SMC was determined.

Materials and methods

Chemicals and materials

Recombinant mouse interferon γ (mIFN-γ; molecular weight ~19 kD; specific activity ~ 10^7 U/mg), minimum essential medium (modified) with Earle's salts (EMEM), RPMI 1640, Iscove's medium, fetal bovine serum (FBS), gentamicin, penicillin, streptomycin, fungizone and 10x phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} were obtained from Gibco BRL (Paisley, UK). Lipopolysaccharide (LPS) from E.Coli 0111.B4 (stimulation indices 14.2 at 31.3 μg/ml and 23.2 at 250 μg/ml), Lucifer yellow CH and bovine serum albumin (BSA) fraction V were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's 'A' PBS was from Oxoid Ltd. (London, UK). Trypsin 1:250 was from Difco (Detroit, MI). Dulbecco's modification of minimum essential medium (DMEM) was from ICN Biomedicals (Costa Mesa, CA). Ethylenediaminetetraacetic acid disodium salt (EDTA) was from Janssen Chimica (Beerse, Belgium). Lithium chloride, glutamine and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were purchased from Merck (Darmstadt, Germany). Percoll was obtained from Pharmacia (Uppsala, Sweden). Six-well plates, six-well size Transwell-COL cell culture chamber inserts (pore size 0.4 μm) and 75 cm^2 cell culture flasks were purchased from Costar Europe Ltd. (Badhoevedorp, Netherlands). 25 cm^2 cell culture flasks were from Nunc A/S (Roskilde, Denmark). Glass coverslips were from Rofa-Mavi (Beverwijk, Netherlands). Cell scrapers and 35 mm culture dishes were supplied by Greiner B.V. (Alphen a/d Rijn, Netherlands).

Human SMC

Primary human SMC were isolated from arteries of umbilical cords by an explant technique. After careful removal of the adventitia, the arterial tissue was cut into small pieces. Explants were incubated in EMEM supplemented with 10% FBS, 50 μg/ml gentamicin and 1.25 μg/ml fungizone (EMEM+++) in a 37°C, 5% CO_2 humidified atmosphere. Cells were allowed to grow for 3-4 weeks until subculturing. These SMC displayed the well known 'hills and valleys' pattern after reaching confluence (243).

Human monocyte-macrophages

Human peripheral blood mononuclear cells were isolated from citrate anti-

coagulated blood by centrifugation over Percoll (specific gravity 1.078 g/cm^3 at 20°C, 290 mOsm; 1000xg, 20 min.) Cells from the interface were collected and platelets, monocytes and lymphocytes were subsequently purified by elutriation essentially as described by De Boer and Roos (282) with minor modifications. The monocytes were further purified by a second Percoll gradient to a purity of about 85%. The remaining contaminating cells were removed by adhering the monocytes to a flask at 37°C in a cabinet for 1 h. The non-adhering cells were removed and the monocytes were then cultured in Iscove's medium (with 10% FBS, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.4) for 1 day at a concentration of 2 - 5 x 10^6 / ml in 25 cm^2 flasks. Then, RPMI 1640, supplemented with 10% FBS and 50 μg/ml gentamicin was added to the cells. After at least 5 days of culturing in 25 cm^2 cell culture flasks, during which the originally rounded cells spreaded, cells were detached from the flask by 15 min incubation at 37°C with an 1:1 mixture of 10 mM EDTA in PBS (without Ca^{2+} and Mg^{2+}), pH 7.1 and EMEM supplemented with 10% FBS and 50 μg/ml gentamicin (EMEM++). Thoroughly pipetting released most of the cells which were collected and centrifuged for 5 min at 400xg. The pellet was resuspended in EMEM++ and $\approx$3.4 x 10^5 cells were plated onto Transwell-Col pore membrane inserts. After 1-6 days, indirect co-culture experiments were initiated with these human monocyte-macrophage cultures.

J774A.1 murine macrophages

The J774A.1 murine monocyte-macrophage cell line (TIB 67) was obtained from the American Type Culture Collection (Rockville, MD). This cell line was adapted to culture from an oil-induced tumor which arose in a BALB/c mouse and displays a number of mature macrophage properties (283,284). J774A.1 cells were cultured in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin (DMEM++). Subcultures were prepared by scraping.

Indirect co-culture system

Transwell-COL chamber inserts were used according to the instructions of the manufacturer. Briefly, 2.6 ml culture medium was added to the cluster plate wells. Hereafter, the 24.5 mm diameter membrane inserts were added. Finally, 1.5 ml culture medium was added to these inserts (for schematic representation see Fig. 5.1).

Murine macrophages: 2-3 x 10^5 J774A.1 cells were seeded on the collagen-treated, microporous membrane inserts in DMEM++ (day 1). After adherence of these cells ($\sim$2 h), the culture medium was replaced by fresh DMEM++ or by DMEM++ containing 1 μg/ml LPS or 100 U/ml mIFN-γ. After 24 h of incubation (day 2), these murine macrophages were carefully washed with PBS (Oxoid): the medium from the

upper compartments was removed and the empty inserts were placed in cluster plate wells containing 2.6 ml PBS. Then, 1.5 ml PBS was added to the inserts. This procedure was repeated with a fresh solution of PBS in both upper- and lower compartment. Thus, both sides of the inserts were 'rinsed' twice this way. At this point, co-culturing started. Glass coverslips with confluent human SMC (passage 2-7) were placed on the bottom of the cluster plate (lower compartment). DMEM++ was added to these cells. Then, the inserts with J774A.1 cells were hung above the SMC. Finally, DMEM++ was added to the inserts. In incubations without macrophages, 'blank' inserts containing only DMEM++ were hung above the SMC. After a 24 h co-culture of SMC and J774A.1 cells, GJIC between SMC was measured (day 3). Just prior to GJIC measurement, the insert was removed from the cluster plate, after which the coverslip containing SMC was transferred to a 35 mm culture dish. From the lower compartment, 1.5 ml culture medium was added to these SMC. HEPES buffer (pH 7.4) was added to these cells (final concentration 20 mM) to stabilize pH during microinjection and fluorescence microscopy. Some co-culture experiments were carried out for two days only. In these '2-days experiments' which involved only untreated macrophages, 5 - 7.5 x 10^5 J774A.1 cells were seeded at day 1. After adherence ($\sim$ 2 h), the inserts with macrophages were placed above the SMC and fresh DMEM++ was added to the co-culture. After 24 h GJIC between SMC was measured. The results of the '2-days' and '3 days experiments' were very similar. Therefore, these results were put together in table 5.1. Experiments in DMEM supplemented with gentamicin and 0.5% BSA were carried out as well.

Human monocyte-macrophages: $\sim$3.4 x 10^5 human monocyte-macrophages on membrane inserts were provided with EMEM++, or with EMEM++ containing 1 μg/ml LPS (day 1). After 24 h of incubation (day 2), these cells were carefully washed with PBS, analogous to the procedure described for J774A.1 cells. Then, co-culture of the human monocyte-macrophages and SMC in EMEM++ started. After 24 h of incubation (day 3), GJIC between SMC was measured.

Measurement of GJIC

GJIC was determined after microinjection of a 20% Lucifer yellow CH (in 0.33 M lithium chloride) solution in a single cell (223). In each SMC culture at least 20 individual cells were microinjected using a vertical injection system (Olympus Injectoscope IMT-2-syf) (224) with a dye filled capillary glass tip (Clark, Pangbourne, UK). The capillary glass tip was prepared using an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of 1 μm. The Lucifer yellow CH filled cells were checked with phase-contrast and fluorescence microscopy directly after microinjection. The number of communicating SMC was determined $\sim$ 15 min after the first injection. The average

number of fluorescent cells was calculated for each incubation. The average number of communicating cells in control incubations was taken as 100% GJIC. Each experiment was performed in duplicate. At least two but predominantly three or more independent tests were done. Data presented in table 5.1 and 5.2 are the average values of the performed tests.

Statistics

Statistical analyses of the data were performed using Students *t*-test ($P \leq 0.05$).

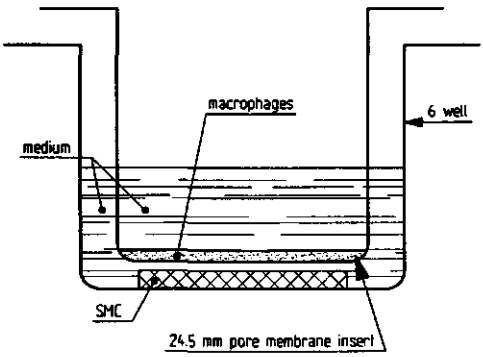

Fig. 5.1. Schematic representation of the Transwell-COL co-culture system. Macrophages are cultured on microporous membrane inserts (upper compartment), whereas SMC are cultured on glass coverslips in the lower compartment.

Results

GJIC between human SMC

The human SMC used in these experiments displayed a distinct GJIC. Lucifer yellow spread over an average of 47 - 94 cells in control incubations. In experiments without FBS, SMC displayed a 20% reduced GJIC as compared to experiments in which 10% FBS was added to the culture medium (data not shown).

GJIC between SMC in indirect co-cultures with J774A.1 cells

In an indirect co-culture system, unstimulated J774A.1 cells were able to reduce GJIC significantly between human SMC by ~22% (table 5.1), both in experiments with or without 10% FBS. The precise amount of J774A.1 cells present on the membrane

inserts did not seem to be of great importance (data not shown). When J774A.1 cells were pretreated with 1 μg/ml LPS or 100 U/ml mIFN-γ, no difference in GJIC between SMC was observed anymore as compared to control incubations without macrophages. Stimulation of J774A.1 cells with either LPS or mIFN-γ apparently levelled out the reduction of GJIC by unstimulated macrophages.

Table 5.1

GJIC (% of control) between human SMC in an indirect co-culture system with J774A.1 murine macrophages

10% FBS	pretreatment J774A.1	J774A.1	GJIC (% of control)
-	-	-	100 ± 1.1
-	-	+	77.3 ± 3.1*
+	-	-	100 ± 2.7
+	-	+	78.7 ± 2.7*
+	LPS	+	97.6 ± 5.9
+	mIFN-γ	+	95.2 ± 3.2

Human SMC were co-cultured with J774A.1 cells for 24 h. In some experiments, J774A.1 cells were pretreated with 1 μg/ml LPS or 100 U/ml mIFN-γ for 24 h. *: significantly different from control incubation.

GJIC between SMC in indirect co-cultures with human monocyte-macrophages

Unstimulated, co-cultured human monocyte-macrophages did not seem to affect GJIC between SMC (table 5.2 A). However, when these monocyte-macrophages were pretreated with 1 μg/ml LPS, a substantial and significant ~60% increase in GJIC between co-cultured SMC was observed (table 5.2 A). However, the extent of stimulation of GJIC between SMC by LPS-pretreated human monocyte-macrophages varied considerably (table 5.2 B). Nevertheless, these experiments clearly demonstrated that activation of these monocyte-macrophages alters their interaction with co-cultured SMC.

Table 5.2
GJIC (% of control) between human SMC in an indirect co-culture system with human monocyte-macrophages

		A	B
pretreatment macrophages	macrophages	GJIC (% of control)	GJIC (% of control)
-	-	100 ± 6.5	
-	+	102.6 ± 4.5	
LPS	+	160.7 ± 29.3*	181.4 ± 0.9
			140.0 ± 2.0

Human SMC were co-cultured with human monocyte-macrophages for 24 h. In some experiments, human monocyte-macrophages were pretreated with 1 μg/ml LPS for 24 h. *: significantly different from control incubation.

Discussion

To our knowledge, this study demonstrates for the first time that cells cultured on pore membrane inserts (macrophages) modulate GJIC between a second cell type (SMC) co-cultured in Transwell-COL cell culture chambers. Since these results were obtained in an indirect co-culture system in which direct cell-cell contact is prevented, it is hypothesized that soluble factors released by macrophages may be involved in this modulation of GJIC between SMC.

Co-culturing of J774A.1 macrophages with human SMC in this system reduced GJIC between SMC with ~22%. However, when J774A.1 cells were stimulated by pretreatment with LPS or mIFN-γ, the reduction in GJIC between co-cultured SMC was not observed anymore. Thus, stimulation of J774A.1 cells by either LPS or mIFN-γ altered the interaction between SMC and these murine macrophages in this indirect co-culture system. It is not known whether stimulation of J774A.1 cells results in the loss of production of GJIC inhibiting substances or whether stimulation of these cells induces the synthesis and secretion of factors that antagonize the action of GJIC inhibiting substances produced constitutively by this cell line.

Unstimulated human monocyte-macrophages did not seem to affect GJIC between

SMC in this Transwell-COL co-culture system. A substantial ~60% increase in GJIC between co-cultured SMC was observed however upon stimulation of these monocyte-macrophages by LPS. Analogous to the indirect co-culture experiments with LPS-treated J774A.1 cells, GJIC between SMC was elevated upon incubation with LPS-stimulated human monocyte-macrophages as compared to incubations with unstimulated macrophages. In experiments with LPS-stimulated murine macrophages, a control level of GJIC was observed, whereas in experiments with LPS-stimulated human monocyte-macrophages GJIC significantly increased as compared to control levels. It is unlikely that traces of LPS, in case of insufficient washing procedures, were responsible for the observed effects on GJIC, since LPS did not affect GJIC between human umbilical vein SMC (285). Furthermore, an LPS-induced down-regulation of heterologous GJIC between human umbilical vein SMC and endothelial cells was reported by these authors.

Macrophages may secrete a complex mixture of growth factors, cytokines, enzymes, cytokine- and enzyme inhibitors, extracellular matrix and other binding proteins, bioactive lipids, hormones, reactive oxygen and nitrogen intermediates, complement components and coagulation factors (286). We can only speculate about the nature of the factors involved in GJIC modulation. The ~22% reduction in GJIC between SMC seen upon co-culturing with unstimulated J774A.1 cells may have been caused by the release of cytokines like tumor necrosis factor α (TNFα) or IFN-γ by these macrophages, since these cytokines have been demonstrated to reduce GJIC between SMC substantially (242,254). The observation that unstimulated human monocyte-macrophages did not affect GJIC between SMC may suggest that no GJIC inhibiting substances were produced by these cells. Alternatively, a cocktail of factors may have been produced by these human monocyte-macrophages in which factors may counteract each others effects on GJIC.

LPS stimulation of J774A.1 cells has been found to induce nitric oxide (NO) production (287,288). NO production, however, is not likely to be the cause of increased GJIC between SMC upon incubation with LPS-treated macrophages as compared to incubations with unstimulated cells, since 1) NO has been suggested to block gap junction channels between tracheal SMC rather than to stimulate GJIC (289) and 2) human inducible nitric oxide synthethase is not inducible by LPS (290).

Another explanation for the effects of stimulated macrophages on GJIC between SMC may be the production of reactive oxygen species by J774A.1 cells and human monocyte-macrophages upon stimulation with LPS or IFN-γ (291,292). However, previous studies (242, chapter 4) suggested that reactive oxygen species inhibited rather than stimulated GJIC between SMC , which renders a role for reactive oxygen species as a cause for increased GJIC rather unlikely.

It is known that treatment of J774.1 cells or other macrophages with LPS may induce TNFα production by these cells (293-296). TNFα production however, does not explain the observed increase in GJIC since previous work clearly demonstrated that this cytokine reduces GJIC between SMC as we already mentioned (242). Other cytokines or growth factors may be better candidates. Basic fibroblast growth factor (bFGF) for instance is able to significantly increase GJIC between SMC (254). It may well be that bFGF-like factors are produced by (LPS) stimulated macrophages.

Alternatively, (J774A.1) macrophages may affect the co-cultured SMC by the release of enzymes. Release of plasmin or heparanase (297,298) may free bFGF and other matrix-associated growth factors from their binding sites in the SMC extracellular matrix (299,300), which in turn may affect GJIC. The release of matrix metalloproteinases like collagenases, gelatinases and stromelysins by macrophages (301,302) may affect the SMC extracellular matrix and SMC characteristics as well. LPS or IFN-γ stimulation of the macrophages may alter the biosynthesis of these metalloproteinases in these cells (301-304), although the effects of LPS and IFN-γ stimulation on for instance gelatinase production differ. Alternatively, the production of cytokines and growth factors by macrophages may induce metalloproteinase expression by SMC themselves (305,306).

The variation in result observed in two separate 'LPS-experiments' with human monocyte-macrophages may originate from small differences in the 'age' of these monocyte-macrophages or from differences in plating efficiency of the monocyte-macrophages upon detachment with EDTA and adherence to the Transwell-COL membrane inserts. The exact number and differentiation state of the monocyte-macrophages may influence the amount of substances (like for instance growth factors and enzyme(s)) produced by these stimulated cells. However, the variation in results may also originate from differences in SMC cultures which may for instance stem from variation in SMC passage number, variation in SMC phenotype, differences in cell density and time of subculturing until confluency. This SMC heterogeneity may affect the type and amount of growth factors that become sequestered in the SMC matrix and may therefore influence the amount of growth factors that are released upon degradation of this extracellular matrix.

Further research should be directed at studying the effect of different types of macrophages on GJIC between SMC. Heterogeneity in atheroma macrophages exist (307,308); the most noticeable difference being the presence of 'normal' macrophages and the presence of macrophage-derived foam cells. It is likely that lipid laden macrophages display a deviant endocytic and secretory repertoire (309) which may affect GJIC between SMC in another way than 'normal' macrophages. Furthermore, macrophages should be exposed to different (patho-)physiological agents with relevance to the process of athero-

sclerosis, in order to study their effects on SMC GJIC in even more detail.

Another way by which macrophages may influence SMC functioning is via direct cell-cell contact, for instance by heterologous GJIC. Heterologous GJIC between SMC and macrophages may be of importance in the pathogenesis of atherosclerosis by facilitating efficient exchange of (reactive) substances between these cell types. The strong expression of connexin43 (Cx43) mRNA in macrophage foam cells in sections of human atherosclerotic carotid arteries (80) and the upregulation of Cx43 protein expression in macrophage foam cells of hypercholesterolemic rabbits (81) suggests that heterologous GJIC may be relevant *in vivo*. Possible effects of for instance growth factors, cytokines, extracellular matrix components, oxidized LDL and other atherogenic chemicals on heterologous GJIC *in vitro*, in addition to functional characterization *in vivo* deserve some attention. As a start, preliminary data from our laboratory suggest that direct, heterologous cell-cell contact, measured as Lucifer yellow transfer from human SMC to co-cultured J774A.1 macrophages (both cell types have been demonstrated to express Cx43; 63,64,310) increases upon incubation with TNFα.

Despite the fact that a lot of detailed questions still have to be elucidated, the conclusion can be drawn that macrophages are important modulators of SMC functioning. Direct and indirect co-culture systems, featuring GJIC modulation of these cell types in incubations with endogenous and/or exogenous compounds, await further study.

Acknowledgement

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Chapter 6
Summary and Concluding Remarks

In this thesis, the effect of leukocyte-derived growth factors and cytokines on GJIC between SMC was investigated. GJIC is regarded as an important mechanism in the control of cell growth, cell differentiation and tissue homeostasis. Disturbance of SMC growth control is regarded to be a key event in the pathogenesis of atherosclerosis in which growth factors and cytokines are thought to play a central role. In the present study, cultured human SMC were incubated with (human) recombinant growth factors and cytokines. TNFα, IFN-γ, PDGF, bFGF and IL-6 were chosen as representatives of several classes of growth modulating factors. These growth factors and cytokines are known to be products of macrophages and/or T lymphocytes and have been detected in human atherosclerotic lesions. After an incubation period, GJIC between SMC was measured. In addition, human SMC were co-cultured with J774A.1 murine macrophages or human monocyte-macrophages in the Transwell-COL cell culture system, to account for the complexity of macrophage secretion patterns. After removal of the macrophages, GJIC between the co-cultured SMC was determined.

The experiments described in **chapter 2 and 3** clearly demonstrate that all factors tested reduced GJIC between SMC with ~20 - 50%, except for bFGF which strongly increased GJIC. Furthermore, these experiments revealed that effects of growth factors and cytokines on GJIC are not univocal and thus cannot be generalized. PDGF, IL-6 and bFGF caused transient effects on GJIC, whereas in experiments with TNFα or IFN-γ, a persistent inhibition of GJIC was obtained.

The most remarkable result of the study described in **chapter 4** was that upon combining TNFα and IFN-γ, GJIC between SMC strongly reduced (up to 86%) in an additive or synergistic manner. Upon long term incubation with the combination of TNFα and IFN-γ, some SMC did not communicate with neighbouring cells at all. This may result in an escape from growth control mechanisms, which, in turn, may lead to disturbance of SMC proliferation, a key event in atherosclerosis. In incubations with other combinations of growth factors and cytokines, (antagonistic) interactive effects on GJIC were observed.

The present investigation provided evidence that reactive oxygen species may play a role in cytokine-induced inhibition of GJIC between SMC. Experiments described in **chapter 2** revealed that pretreatment of SMC with antioxidants like ascorbic acid, α-tocopherol or

GSH prevented the inhibition of GJIC upon exposure of SMC to TNFα. Studies with SOD (**chapter 4**) demonstrated that the superoxide radical may be involved in GJIC reduction by TNFα, since incubation with SOD, even hours after the addition of TNFα, restored GJIC to control values. Furthermore, SOD partly restored IFN-γ effects on GJIC in the short - but not in the long term. When SMC were incubated with TNFα and IFN-γ simultaneously for 24 h, high levels of SOD could not even partly counteract the strong inhibition of GJIC caused by these cytokines. Thus, other, superoxide-unrelated mechanisms may affect GJIC more predominantly in long term incubations with the combination of TNFα and IFN-γ. One such mechanism may be represented by the reduced Cx43 staining which was observed in immunofluorescence studies on SMC cultures incubated with these cytokines (**chapter 4**), which may be an indication for the reduced presence of functional gap junction channels.

PDGF-AA, PDGF-BB, IL-6, IFN-γ, TNFα and bFGF all stimulated SMC proliferation in our cell culture system, as individual factors as well as in combinations (**chapter 3 and 4**). Upon comparing these cell proliferation results with GJIC data, a complex relationship between modulation of GJIC, cell proliferation and the process of atherosclerosis is suggested.

Experiments described in **chapter 5** demonstrated that macrophages cultured on pore membrane inserts modulate GJIC between SMC co-cultured in Transwell-COL cell culture chambers. Since these results were obtained in an indirect co-culture system which prevents direct cell-cell contact, it was hypothesized that soluble factors, released by macrophages, may be involved in the modulation of GJIC between SMC. At this moment, one can only speculate about the nature of the factors involved in this macrophage-dependent modulation of GJIC. The results clearly indicate that the source and activation state of macrophages were of importance in these co-culture experiments. Therefore, further research should be aimed at studying the effect of different types of macrophages on GJIC between co-cultured SMC. Heterogeneity in atheroma macrophages exists; the most noticeable difference being the presence of 'normal' macrophages and the presence of macrophage-derived foam cells, which are likely to differ in endocytic and secretory repertoire. Furthermore, macrophages should be exposed to different (patho-)physiological agents with relevance to the process of atherosclerosis, in order to study their effects on GJIC between SMC in even more detail.

The present study provides a good starting point for further research aimed at the understanding of mechanisms by which environmental contaminants or drugs might interfere with atherogenesis. It is already known that widespread food chain and cigarette smoke contaminants like for example benzo[a]pyrene, polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin may affect the pathogenesis of atherosclerosis in several ways,

for instance by damaging SMC DNA (311), disrupting endothelial barrier function (312), or by modulation of plasma cholesterol and lipoprotein levels (313,314). Furthermore, chemicals like components in cigarette smoke condensate may modulate GJIC between SMC (57). Considering that growth factors and cytokines like TNFα and IFN-γ may have marked effects on GJIC, one may assume that environmental contaminants and drugs capable of affecting the expression of growth factors and cytokines (315-329) or their receptors (325,326,330-332) may interfere with GJIC in an indirect manner. In the case of atherogenesis for instance, chemicals may stimulate growth factor- and cytokine production by SMC and/or macrophages which, in turn, may influence homologous GJIC between SMC. In addition, exogenous chemicals may influence heterologous GJIC between macrophages and SMC as well; either directly, or indirectly via the induction of growth factor and cytokine expression by these cells. As a consequence, macrophage-derived reactive substances will have more - or just less- impact on SMC functioning.

Relatively short exposures to environmental contaminants or drugs in individuals in which plaques have already passed some critical phases in the atherosclerotic process might enhance the severity of the lesions. Further research along this line may also lead to the identification of nutritional or chemical factors that may have beneficial (protective / regressive) effects on the development of atherosclerotic lesions.

Modulation of GJIC by growth factors and cytokines may affect a response-to-injury. On the other hand, modulation of GJIC may also play a role in the monoclonal expansion of cells. Therefore, the response-to-injury hypothesis and the monoclonal theory may be compatible is some respects, as was previously suggested by Zwijsen (333).

The results of the present study may also be applicable to other pathophysiological phenomena, in which growth factors and cytokines may play a prominent role in the onset or progression of the disease. Proliferative diseases like pulmonary fibrosis, glomerulosclerosis and liver cirrhosis share some pathobiologic mechanisms with atherosclerosis, including leukocyte infiltration, mesenchymal cell proliferation and enhanced matrix synthesis (334-337). Leukocyte-derived growth factors and cytokines may modulate GJIC between the mesenchymal cells concerned, which in turn may result in abnormal cell proliferation. It is known that certain chemicals may contribute to the development of these diseases. The mechanisms by which these chemicals act may be linked to the processes studied and discussed in this thesis.

Overall, the information presented in this thesis concerning the possible role of growth factors and cytokines in the pathophysiology of atherosclerosis provides a useful instrument to study possible modulatory effects of chemicals on the process of atherosclerosis via the

mechanisms mentioned above.

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Abbreviations

Balb C 3T3	mouse embryonic cell line
BE	normal human bronchial epithelial cells
BEAS-2B	adenovirus 12-SV40 hybrid virus transformed BE cells
bFGF	basic fibroblast growth factor
BME	bovine microvascular endothelial cells
BMP	bone morphogenetic protein
BSA	bovine serum albumin
C3H/10T1/2	mouse embryonic fibroblast cell line
cAMP	cyclic adenosine monophosphate
Cx	connexin
D	dalton
DMEM	Dulbecco's modification of minimum essential medium
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid (disodium salt)
EGF	epidermal growth factor
EMEM	Eagle's minimum essential medium (modified) with Earle's salts
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GJIC	gap junctional intercellular communication
GM-CSF	granulocyte-macrophage colony stimulating factor
GSH	reduced glutathione
H_2O_2	hydrogen peroxide
h	hour(s)
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HGF	hepatocyte growth factor
HLA	human leukocyte associated antigen
HUVEC	human umbilical vein endothelial cells
IFN-γ	interferon-γ
IL-1α	interleukin-1α
IL-6	interleukin-6
J774A.1	murine monocyte-macrophage cell line
K7	Ni(II)-immortalized human kidney epithelial cells

kD	kilodalton
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MC3T3-E1	osteoblastic cells
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
mIFN-γ	mouse interferon-γ
min	minute(s)
mRNA	messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NADH	nicotinamide adenine dinucleotide
NDFß	neu differentiation factor ß
NHEK	normal human epidermal keratinocytes
NO	nitric oxide
NO_2	nitrite
NOS	nitric oxide synthase
NRK	normal rat kidney cells
NRS	normal rabbit serum
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
SD	standard deviation
SM	smooth muscle
SMC	smooth muscle cell(s)
SOD	superoxide dismutase
T51B	rat liver epithelial cells
TGFß	transforming growth factor ß
TNFα	tumor necrosis factor α
TPA	12-*O*-tetradecanoylphorbol-13-acetate

Samenvatting en Slotopmerkingen

Het onderzoek beschreven in dit proefschrift heeft betrekking op effekten van door leukocyten geproduceerde groeifaktoren en cytokinen op de intercellulaire communicatie via gap junctions (GJIC) tussen gladde spiercellen. GJIC speelt een belangrijke rol bij de regulatie van processen als celdeling, celdifferentiatie en weefselhomeostase. Verstoring van normale gladde spiercelproliferatie mechanismen wordt gezien als een sleutelgebeurtenis bij het ontstaan van atherosclerose, waarbij groeifaktoren en cytokinen een centrale rol toebedacht wordt. In het onderhavige onderzoek werden humane gladde spiercellen blootgesteld aan (humane) recombinante groeifaktoren en cytokinen. TNFα, IFN-γ, PDGF, bFGF en IL-6 werden gekozen als vertegenwoordigers van een aantal klassen van groeimodulerende faktoren. Van deze groeifaktoren en cytokinen is bekend dat zij geproduceerd worden door macrofagen en/of T lymfocyten en dat zij aanwezig zijn in humane atherosclerotische lesies. Na een incubatieperiode werd de GJIC tussen de blootgestelde gladde spiercellen gemeten. Tevens werden humane gladde spiercellen samen met J774A.1 muis macrofagen of humane monocyt-macrofagen gecultiveerd in het Transwell-COL kweeksysteem, zodat de complexiteit van macrofaag secretiepatronen tot uitdrukking kon komen. Na het verwijderen van de macrofagen uit het kweeksysteem werd de GJIC tussen de achtergebleven gladde spiercellen bepaald.

De experimenten beschreven in **hoofdstuk 2 en 3** tonen duidelijk aan dat alle geteste faktoren GJIC tussen gladde spiercellen reduceren met ~20 - 50%, behalve bFGF die GJIC sterk doet toenemen. Deze experimenten laten verder zien dat de effekten van groeifaktoren en cytokinen op GJIC niet eenduidig zijn, zodat ze niet veralgemeniseerd kunnen worden. PDGF, IL-6 en bFGF moduleren GJIC tijdelijk, terwijl in experimenten met TNFα of IFN-γ een aanhoudende remming van GJIC werd verkregen.

Het meest opvallende resultaat van de studie beschreven in **hoofdstuk 4** was dat een combinatie van TNFα en IFN-γ GJIC tussen gladde spiercellen op een additieve of synergistische wijze (met maximaal 86%) sterk reduceerde. Sommige gladde spiercellen communiceerden in het geheel niet meer met naburige cellen wanneer zij gedurende langere tijd blootgesteld werden aan de combinatie van TNFα en IFN-γ. Hierdoor zouden deze cellen kunnen ontsnappen aan groeicontrole mechanismen, wat weer kan resulteren in een verstoring van de normale gladde spiercelproliferatie, een sleutelgebeurtenis in het atherosclerotische proces. In incubaties met andere combinaties van groeifaktoren en cytokinen werden (antagonistische) interactieve effekten op GJIC waargenomen.

Het onderhavige onderzoek leverde aanwijzingen op dat reactieve zuurstof species een rol kunnen spelen in de cytokine-geïnduceerde remming van GJIC tussen gladde spiercellen. De experimenten die beschreven staan in **hoofdstuk 2** laten zien dat voorbehandeling van gladde spiercellen met antioxidantia als ascorbinezuur, α-tocopherol of GSH de remming van GJIC kon voorkomen wanneer deze cellen vervolgens blootgesteld werden aan TNFα. Studies met SOD (**hoofdstuk 4**) tonen aan dat het superoxide radicaal betrokken lijkt te zijn bij de GJIC remming door TNFα, omdat blootstelling aan SOD, zelfs uren na TNFα toevoeging, GJIC deed herstellen tot controle waarden. SOD was tevens in staat om IFN-γ geïnduceerde effekten op GJIC ten dele teniet te doen in kortdurende experimenten, een eigenschap die bij langdurige IFN-γ incubaties niet meer tot uitdrukking kwam. Een hoge concentratie SOD bleek niet in staat te zijn de sterke GJIC remming die veroorzaakt werd door een 24 uurs blootstelling aan een combinatie van TNFα en IFN-γ zelfs maar gedeeltelijk op te heffen. Derhalve lijken voornamelijk andere, superoxide-onafhankelijke mechanismen GJIC te beïnvloeden in de langer durende incubaties met de combinatie van TNFα en IFN-γ. Een dergelijk mechanisme kan verband houden met de verminderde Cx43 kleuring die werd waargenomen in immunofluorescentie studies die werden uitgevoerd met gladde spiercellen die aan deze cytokinen blootgesteld waren (**hoofdstuk 4**), wat wellicht duidt op een verminderde aanwezigheid van functionele gap junction kanalen.

In ons kweeksysteem bleken PDGF-AA, PDGF-BB, IL-6, IFN-γ, TNFα en bFGF in staat om gladde spiercelproliferatie te induceren, zowel als individuele faktoren als in diverse combinaties (**hoofdstuk 3 en 4**). Door het vergelijken van de celproliferatie resultaten en de GJIC gegevens lijkt de conclusie gerechtvaardigd dat er een complex verband bestaat tussen modulatie van GJIC, celproliferatie en het proces van atherosclerose.

De experimenten beschreven in **hoofdstuk 5** laten zien dat macrofagen die gecultiveerd werden op porie-bevattende membranen in staat bleken GJIC tussen gladde spiercellen die zich in hetzelfde Transwell-COL kweeksysteem bevonden te moduleren. Daar deze resultaten verkregen werden in een kweeksysteem waarbij direkt cel-cel contact niet mogelijk was, werd geconcludeerd dat oplosbare faktoren, afgescheiden door de macrofagen, betrokken waren bij deze modulatie van GJIC tussen gladde spiercellen. Op dit moment kan er slechts gespeculeerd worden over de aard van de faktoren die verantwoordelijk zijn voor deze macrofaag-afhankelijke modulatie van GJIC. De resultaten tonen duidelijk aan dat de oorsprong en de activatie toestand van de macrofagen van belang waren in de experimenten. Vervolgonderzoek zou derhalve gericht moeten zijn op het bestuderen van de effekten die verschillende typen macrofagen zouden kunnen hebben op GJIC tussen gladde spiercellen. Atheroma-macrofagen zijn heterogeen van

samenstelling; het meest opvallende verschil betreft de aanwezigheid van 'normale' macrofagen naast de macrofaag-schuimcellen die er een ander endocytose- en secretiepatroon op na houden. Macrofagen zouden bovendien blootgesteld kunnen worden aan diverse (patho)fysiologische stimuli die relevant zijn voor het proces van atherosclerose, zodat macrofaag-effekten op GJIC tussen gladde spiercellen in nog meer detail bestudeerd kunnen worden.

Het onderzoek beschreven in dit proefschrift vormt een goed uitgangspunt voor nader onderzoek naar mechanismen waarlangs milieuvervuilende stoffen of medicijnen zouden kunnen interfereren met de atherogenese. Het is bekend dat veelvoorkomende contaminanten in sigarettenrook en in de voedselketen, zoals bijvoorbeeld benzo[a]pyreen, polychloorbifenylen en 2,3,7,8-tetrachloordibenzo-*p*-dioxine, de pathogenese van atherosclerose op verschillende manieren kunnen beïnvloeden; onder andere door het DNA van gladde spiercellen te beschadigen (311), door de endotheliale barrière te ontwrichten (312) of door de plasmacholesterol- en lipoproteïne concentraties te veranderen (313,314). Chemicaliën zoals componenten in sigarettenrookcondensaat kunnen bovendien GJIC tussen gladde spiercellen moduleren (57). Het lijkt redelijk te veronderstellen dat omgevingsfaktoren en medicijnen die in staat zijn de expressie van groeifaktoren en cytokinen (315-329) of hun receptoren (325,326,330-332) te beïnvloeden, GJIC op een indirekte wijze kunnen moduleren, in beschouwing nemende dat groeifaktoren en cytokinen als TNFα en IFN-γ uitgesproken effekten op GJIC kunnen hebben. In de atherogenese bijvoorbeeld zouden chemicaliën de groeifaktor- en cytokineproduktie door gladde spiercellen en/of macrofagen kunnen stimuleren welke op hun beurt de homologe GJIC tussen gladde spiercellen kunnen beïnvloeden. Exogene chemicaliën zouden bovendien ook de heterologe GJIC tussen macrofagen en gladde spiercellen hetzij direkt, hetzij indirekt via de inductie van groeifaktor- en/of cytokine-expressie door deze cellen kunnen beïnvloeden. De consequentie hiervan zou kunnen zijn dat reactieve verbindingen, geproduceerd door macrofagen, meer - of juist minder - invloed op het functioneren van gladde spiercellen kunnen uitoefenen.

In individuen waarin plaques al enkele kritische fasen van het atherosclerotische proces doorlopen hebben zouden relatief kortdurende blootstellingen aan milieuvreemde stoffen of medicijnen de ernst van de lesies kunnen doen toenemen. Verder onderzoek langs deze weg kan mogelijk ook resulteren in de identificatie van voedingscomponenten en chemische stoffen die juist een heilzaam (beschermend / regressief) effekt op de ontwikkeling van atherosclerotische lesies hebben.

Modulatie van GJIC door groeifaktoren en cytokinen kan een 'response-to-injury'

beïnvloeden. Modulatie van GJIC kan echter ook een rol spelen bij de monoclonale expansie van cellen. Derhalve kunnen de 'response-to-injury' hypothese en de monoclonale theorie verenigbaar zijn op een aantal punten, zoals Zwijsen reeds eerder suggereerde (333).

De resultaten van het onderzoek beschreven in dit proefschrift kunnen wellicht ook toegepast worden bij andere pathofysiologische processen waarbij groeifaktoren en cytokinen een belangrijke rol spelen bij het ontstaan of verloop van de ziekte. Proliferatieve ziekten als longfibrose, glomerulosclerose en levercirrhose delen een aantal pathobiologische mechanismen met atherosclerose, zoals leukocyt infiltratie, mesenchymale celproliferatie en verhoogde matrix synthese (334-337). De door leukocyten geproduceerde groeifaktoren en cytokinen zouden GJIC tussen de mesenchymale cellen kunnen moduleren, wat weer zou kunnen resulteren in een abnormale celproliferatie. Het is bekend dat bepaalde chemicaliën bijdragen aan de ontwikkeling van deze ziekten. De mechanismen waarlangs deze chemicaliën hun invloed uitoefenen zouden verband kunnen houden met de processen die bestudeerd en bediscussieerd zijn in dit proefschrift.

De informatie die in dit proefschrift gepresenteerd is betreffende de mogelijk rol van groeifaktoren en cytokinen in de pathofysiologie van atherosclerose vormt een nuttig instrument om mogelijke modulatoire effekten van chemicaliën op het proces van atherosclerose via de hierboven genoemde mechanismen te bestuderen.

List of Publications

- De Waal, A., A. Vaz Gomes, A. Mensink, J.A. Grootegoed and H.V. Westerhoff. 1991. Magainins affect respiratory control, membrane potential and motility of hamster spermatozoa. *FEBS Letters* 293:219-223.

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- Mensink, A., T. van der Wijk, I. Struik, L.H.J. de Haan, A. Brouwer and J.H. Koeman. Interactive effects on gap junctional intercellular communication by human smooth muscle cell mitogens. Submitted.

- Mensink, A., F.P.J. Mul, T. van der Wijk, A. Brouwer and J.H. Koeman. Macrophages influence gap junctional intercellular communication between smooth muscle cells in a co-culture model. Submitted.

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Curriculum Vitae

Anne Mensink werd op 2 januari 1967 geboren in Den Haag. In 1985 behaalde zij daar het VWO diploma aan het Edith Stein College (met lof) en begon zij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. Het propaedeutisch examen werd behaald in september 1986 (met lof). Tijdens de doctoraalfase verrichtte zij onderzoek bij de vakgroepen Toxicologie (prof. dr J.H. Koeman; dr ir R.M.L. Zwijsen), Moleculaire Biologie (prof. dr A. van Kammen; dr R.C. van den Bos) en Experimentele Diermorfologie en Celbiologie (sectie Celbiologie en Immunologie; prof. dr W.B. van Muiswinkel; dr B.M.L. Verburg-van Kemenade). Aansluitend werd een stageperiode doorgebracht op het Nederlands Kanker Instituut te Amsterdam (dr H.V. Westerhoff; dr A. de Waal). De studie werd in augustus 1991 met lof afgerond. In september 1991 startte zij met promotie-onderzoek op een zelf geschreven onderzoeksproject bij de vakgroep Toxicologie van de Landbouwuniversiteit, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd onder leiding van prof. dr J.H. Koeman. Naast het verrichten van onderzoek werden er een aantal modulen van de postdoctorale opleiding Toxicologie gevolgd en werd er onderwijs gegeven aan doctoraalstudenten van de Landbouwuniversiteit en aan cursisten van de postdoctorale opleiding Toxicologie. Vanaf 1 september 1997 is zij werkzaam als hoofd van de afdeling Biotechnologie van Nutricia Corporate Research te Wageningen.