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Expression of Platelet-Derived Growth Factor Isoforms and Their Receptors in Cells Accumulating in the Human Atherosclerotic Lesion

Alexandra Krettek

Göteborg University
Sweden
1999

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I. Krettek A, Fager G, Jernberg P, Östergren-Lundén G, Lustig F.
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Alexandra Krettek

Wallenberg Laboratory for Cardiovascular Research, Department of Heart and Lung Diseases, Göteborg University, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden

ABSTRACT

Atherosclerosis is a progressive and complex disease, which narrows the lumen of arteries and eventually causes cardiovascular complications such as ischaemic heart disease and stroke. Hallmarks of the disease development are; a compromised endothelial barrier, the accumulation of lipids and immunocompetent monocyte-derived macrophages (MDM) and T-cells, the formation of MDM foam cells, and migration and proliferation of medial arterial smooth muscle cells (SMC) in the innermost layer (intima) of the arterial wall. While lipid-lowering therapy favourably retards atherosclerosis progression by reducing the lipid component of intimal thickenings, there is no effective therapy to control cell accumulation in the intima. A key role in this cellular response has been attributed to the Platelet-Derived Growth Factor (PDGF). The main aims of this thesis were to establish a better understanding of the role of PDGF and its receptors (PDGF-R) in the cellular responses in the complex atherosclerotic plaque by studying human SMC and MDM in simplified *in vitro* systems. Specifically, I studied i) if the expressions of PDGF isoforms and their receptors are affected by changes in cell phenotype, ii) if this expression is modulated by exogenous cytokines, and iii) if different PDGF homodimers affect major cellular events in the atherosclerotic lesion; proliferation of SMC and migration of MDM. The results showed that human SMC and MDM expressed PDGF and PDGF-Rs *in vitro*. Both PDGF and PDGF-R genes were independently regulated and showed a complex gene regulatory pattern. The expression of these genes could be influenced by phenotypic changes and cytokines. SMC downregulated PDGF mRNA during dedifferentiation upon serum exposure. In contrast, blood-derived monocytes differentiated to MDM in the presence of serum and increased both PDGF isoform mRNAs, suggesting that PDGF might be important during macrophage differentiation. All tested PDGF homodimers (PDGF-AA_L, -AA_S, and -BB_S) induced SMC proliferation whereas only PDGF-AA_L induced directed migration in early MDM and this was coupled to an increase in PDGF-Rα expression.

Taken together, these results suggest that PDGF-mediated mechanisms may be involved in cell accumulation in atherosclerotic lesions, influencing proliferation of SMC and chemoattraction of early MDM.

Key Words *PDGF • receptor • smooth muscle cell • macrophage • proliferation • migration • cytokine*

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From the Wallenberg Laboratory for Cardiovascular Research
and the Department of Heart and Lung Diseases,
Göteborg University, Sahlgrenska University Hospital

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Alexandra Krettek
MSc

Doctoral Thesis

Göteborg 1999

A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarises the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted, or in manuscript).

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To my parents
Detlef and Evaline

The Role of the Infinitely Small in Nature Is Infinitely Great

Louis Pasteur

(1822-1895)

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PREFACE

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

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ABBREVIATIONS

aFGF	Acidic fibroblast growth factor
Chemokinesis	Random motility
Chemotaxis	Directed migration
Early hMDM	Human monocyte-derived macrophage, cultured in SFM
EC	Endothelial cell
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GAG	Glycosaminoglycan
hASMC	Human arterial smooth muscle cell
hAVEC	Human adult vein endothelial cell
hMDM	Human monocyte-derived macrophage, cultured in SM
IFN-γ	Interferon-γ
IL-6	Interleukin-6
MDM	Monocyte-derived macrophage
PDGF	Platelet-derived growth factor
PDGF-A_L	Long PDGF-A isoform
PDGF-A_S	Short PDGF-A isoform
PDGF-B_L	Long PDGF-B isoform
PDGF-B_S	Short PDGF-B isoform
PDGF-R	Platelet-derived growth factor receptor
PDGF-Rα	Platelet-derived growth factor receptor α-subunit
PDGF-Rβ	Platelet-derived growth factor receptor β-subunit
RT-PCR	Reverse-transcriptase polymerase chain reaction
SFM	Serum-free medium
SM	Serum-containing medium
SMC	Smooth muscle cell
TGF-β	Transforming growth factor-β
VSMC	Vascular smooth muscle cell

INTRODUCTORY REMARKS

Atherosclerosis is a progressive, complex, and multifactorial disease, which narrows the lumen of arteries and eventually causes cardiovascular complications such as ischaemic heart disease and stroke. This narrowing is due to patch-wise thickenings of the innermost layer of the arterial wall (intima) by the accumulation of lipids (i.e. cholesterol), cells, and extracellular matrix (ECM). However, the contribution of each individual component to the progression of the disease varies both between plaques and subjects.

The cholesterol component of the plaque is large in hypercholesterolemia, which explains up to 50 % of subsequent fatal and non-fatal myocardial infarctions in prospective studies (Pooling Project, 1978; Braunwald, 1997). Efficient cholesterol-lowering therapy reduces the incidence of major cardiovascular events by about 30 % (Scandinavian Simvastatin Survival Study (4S), 1994; Shepherd et al., 1995; Sacks et al., 1996; Fager and Wiklund, 1997). Clearly, cholesterol-lowering therapy is efficient in reducing complications of atherosclerosis due to hypercholesterolemia but it is unlikely to influence other components of atherosclerotic plaques in the large numbers of infarction patients with normal cholesterol levels.

Since hypercholesterolemia only explains approximately 50% of subsequent fatal and non-fatal myocardial infarctions, other factors need to be taken into consideration. Up to 50% of the volume of the atherosclerotic wall enlargement consists of arterial smooth muscle cell (ASMC) and ASMC-derived ECM. There is currently no clinically useful therapy to control intimal cell and ECM accumulation. Indeed, our understanding of the mechanisms underlying these processes are insufficient even to suggest therapeutic approaches. Therefore, more research is needed to provide sound bases for therapeutic developments.

Current knowledge about the development of atherosclerosis allows us to conclude that; 1) the endothelial barrier is compromised in atherosclerosis; 2) accumulation of immunocompetent T-cells and monocyte-derived macrophages (MDM) and MDM-derived foam cells is an early event in the progression of the disease (Ross, 1993); 3) migration and proliferation of medial ASMC often contributes to most of the non-lipid plaque volume (Bobik and Campbell, 1993; Ross, 1993).

A compromised endothelium is associated with extravasation of white blood cells, platelet adhesion, and loss of the anticoagulant barrier provided by the

functional endothelium (Holvoet and Collen, 1997). The loss of an intact endothelial surface is regarded as a promoter of atherosclerosis and initiator of thrombosis (Ross and Glomset, 1976b). T-cells and monocytes from the blood infiltrate the intima, leading to the development of an inflammatory reaction. This reaction may be pro-atherosclerotic by increasing apoptosis of vascular SMC (Geng et al., 1996), decreasing collagen synthesis (Amento et al., 1991), or inducing matrix metalloproteinase (MMPs) production (Henney et al., 1991; Galis et al., 1994a; Galis et al., 1994b). However, it may also be anti-atherosclerotic, by affecting MMP secretion (Sarén et al., 1996), inducing nitric oxide production (Yan et al., 1996), or reducing SMC proliferation (Hansson et al., 1991). Initially, MDM may be beneficial in removing accumulated lipoproteins but later they may even be harmful by producing cytokines, free radicals, growth factors, and matrix-degrading enzymes.

ASMC are often the dominating cellular component of the intimal lesion and may constitute as much as half of the plaque volume. Too many ASMC within the intima undoubtedly contribute to luminal narrowing and increased responses to vasoconstrictor stimuli (Folkow, 1987). However, too few ASMC and too little ASMC-derived ECM have been implicated as a cause of plaque vulnerability and rupture by providing too thin or fragile caps over the gelatinous lipid core (Davies et al., 1993; Falk et al., 1995; Fuster et al., 1998).

A key role in intimal hyperplasia has been attributed to the platelet-derived growth factor (PDGF), a well-recognised mitogen for mesenchymal cells, including ASMCs. The main aims of this thesis were to establish a better understanding of the role of PDGF and its receptors (PDGF-Rs) in the cellular responses in the complex atherosclerotic plaque by studies in simplified *in vitro* systems. Increased knowledge of the expression of PDGF isoforms and their receptors may facilitate the development of therapies for a direct control of cell accumulation during plaque development.

THE VESSEL WALL

A healthy/normal arterial wall consists morphologically of three distinct layers (Fig. 1). The innermost layer is the *intima.* This layer consists of ECM and has a barrier of endothelial cells located on the luminal side close to the blood flow. The ECM is an intricate network of macromolecules located between cells. It consists of a variety of proteins and polysaccharides. A layer of elastic fibers, *lamina elastica interna*, determines the border to the *media*, which is the middle portion of the arterial wall. The media consists mainly of diagonally arranged SMCs in a matrix of collagen, elastic fibers, and proteoglycans.

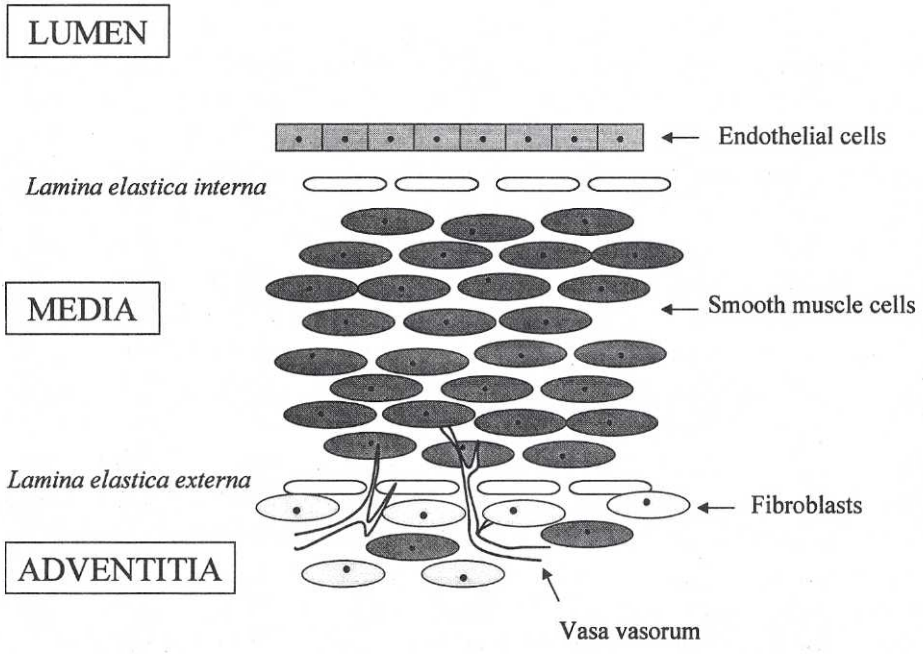

Fig. 1. Cross-section of a normal arterial wall.

The outermost layer of the vessel is the *adventitia* which is often separated from the media by another layer of elastic fibers, the *lamina elastica externa.* The adventitia mainly consists of fibroblasts and loosely arranged SMCs, in between which ECM is interspersed. The adventitia also contains blood capillaries (*vasa vasorum*) which supply the peripheral arterial wall with nutrients and oxygen.

The inner half is supplied by diffusion from the main vessel lumen (Ross and Glomset, 1976a). A gradual decrease in oxygen levels towards the centre of the media has been observed both from the luminal and the adventitial side (Jurrus and Weiss, 1977; Crawford et al., 1980; Crawford and Kramsch, 1988; Santilli et al., 1992). It has been suggested that human atherosclerosis may be initiated by occlusion of the vasa vasorum and concomitant hypoxia, resulting in a hyperplastic lesion composed mainly of SMCs (Barker et al., 1993). Hence, in some cases, atherosclerosis may be a disease of the outer layers of the arterial wall (van der Loo and Martin, 1997.)

Several cell types are found within the arterial wall. They all contribute in their own way to the homeostasis of the normal arterial wall, but also play significant roles in the diseased vessel. A short introduction to the main cell types studied *in vitro* in this thesis is given below.

Endothelial Cells (EC) create a single-layered barrier to the lumen, which regulates molecular and cellular movements across the vessel wall thus creating an adaptable life-support system (Holvoet and Collen, 1997). The normal endothelium constitutes a non-adhesive surface for both platelets and monocytes as well as an anti-coagulant surface. EC produce several important factors such as nitric oxide (NO) (Palmer et al., 1987) and cell adhesion molecules (Cybulsky and Gimbrone, 1991). When the endothelial barrier is compromised or injured, surrounding EC start to spread and migrate into the compromised area (Ross and Glomset, 1976a). This process may elicit the secretion of growth factors and cytokines, which stimulate neighbouring cells (Ross, 1993; Holvoet and Collen, 1997).

Smooth Muscle Cells (SMCs) form diagonally arranged layers of differentiated/ quiescent/contractile cells within the media. Due to exogenous stimuli, such as growth factors and cytokines, they may be induced to change their phenotype. It has been shown that T-cells through the release of IFN-γ induce dedifferentiation (proliferation) of SMCs (Rolfe et al., 1995). SMC dedifferentiation can also be stimulated by macrophages *in vitro* (Rennick et al., 1988). This could be due to macrophage-derived heparan-sulfate degrading enzymes which have been shown to induce the dedifferentiated phenotype in SMCs (Campbell et al., 1992). Proteoglycans in the arterial wall may also affect SMC phenotype. Heparin and heparan-sulfate proteoglycans inhibit phenotypic changes in SMCs thus rendering them in a quiescent/differentiated state (Fager et al., 1988; Fager et al., 1989; Bingley et al., 1998), whereas differential expression of versican (Lemire et al., 1996) and perlecan (Weiser et al., 1996) in SMCs might contribute to different phenotypes. PDGF and/or serum

stimulates SMC to dedifferentiate and proliferate *in vitro* (Fager et al., 1988; Fager et al., 1989; Fager et al., 1992).

Monocytes/Macrophages (MDM). Classically white blood cells are grouped into three main categories on the basis of their appearance in the light microscope; granulocytes, monocytes, and lymphocytes. Together with the neutrophils, macrophages are the main "professional phagocytes" of the body. As such, macrophages digest invading microorganisms and foreign bodies and remove damaged cells from tissues. When monocytes leave the blood stream and enter the vessel wall through the compromised endothelial cell layer, they start to differentiate into macrophages (Daugherty and Rateri, 1993; Ross, 1993). Macrophages are sources of cytokines, growth factors, free radicals, and matrix-metalloproteinases (MMPs). Oxidised low density lipoproteins (LDL) are taken up by differentiated macrophages through the scavenger receptor (Sparrow et al., 1989). This eventually leads to foam cell formation within the vessel wall (Ross, 1993). A lipid core starts to develop within the atherosclerotic plaque, and results in increasing plaque vulnerability.

DEVELOPMENT OF ATHEROSCLEROSIS

The name "atherosclerosis" implies that mature plaques typically consist of two components; the soft, lipid-rich atheromatous "gruel" core (atherosis) and the hard, collagen-rich sclerotic/fibrous tissue cap (sclerosis) (Falk et al., 1995). By deduction from animal studies, it has been suggested that the atherosclerotic lesion progresses through three main stages; 1) fatty streaks, 2) fibrolipid lesions, and 3) complicated lesions (Daugherty and Rateri, 1993) (Fig. 2). Furthermore, all three stages may coexist in the same individual and even within the same artery. The earliest type of lesion, the *fatty streak* (Fig. 2A), may be found also at young age (Napoli et al., 1997). It is mainly an inflammatory lesion, consisting of MDM and T-cells (Stary et al., 1994). The fatty streak develops when monocytes from the blood migrate into the subendothelial space, differentiate into macrophages and start to phagocytose oxidised LDL via the scavenger receptor which converts them to foam cells. The lipid deposition progresses further into the *fibrolipid lesion* (Fig. 2B) by creating an acellular gruel core region. A fibrous cap is formed and stabilised through SMC proliferation, as discussed below. Thrombus and ulceration of atherosclerotic lesions subsequently results in the final stage, a *complicated lesion* (Fig. 2C) (Daugherty and Rateri, 1993). A rupture, often at the shoulder regions of the plaque, eventually leads to thrombotic occlusion and acute clinical complications. This occurs most frequently where the fibrous cap is

thinnest, most heavily infiltrated by foam cells, and therefore weakest (Falk et al., 1995; Fuster et al., 1998). Local MMPs have been implicated in this process.

The first theories about the mechanisms of atherosclerosis appeared in the early 19th century. Two simultaneously occurring hypotheses were the incrustation hypothesis and the lipid hypothesis. The *incrustation hypothesis* (von Rokitansky, 1852) suggested that intimal thickening resulted from fibrin deposition and subsequent fibroblast organisation with the accumulation of lipid being a secondary event. The *lipid hypothesis* (von Virchow, 1856) suggested that lipid accumulated within the arterial wall because the mechanisms of lipid deposition predominated over those of removal. These theories were eventually integrated in the more complex *response-to-injury theory* in 1976 (Bondjers et al., 1976; Ross and Glomset, 1976a). This theory hypothesised that some kind of injury was a prerequisite for the initiation of the disease and that the normal healing procedure went awry due to unfavourable conditions such as hyperlipidemia. The complexity of atherosclerosis became evident when it was realised that the immune system is actively involved in the progression of the disease (Hansson et al., 1989; Libby and Hansson, 1991). In fact, the cellular interactions in atherogenesis are similar to those in other chronic inflammatory-fibroproliferative diseases, such as cirrhosis and rheumatoid arthritis (Ross, 1999). In recent years, the inflammatory component of atherosclerosis has been given much attention. It is now recognised that these events are able to contribute to the disease also without obvious injury to the endothelium. Within the immune system both positive and negative signals may be induced. Details on the immunological mechanisms in the induction of atherosclerosis have been reviewed by Hansson (1997).

A correlation between the incidence of atherosclerosis and the presence of pro-inflammatory microorganisms has been shown. However, there is no direct evidence that these organisms can cause the lesions of atherosclerosis (Libby et al., 1997). Very recently it was shown that both chlamydial and human heat shock protein 60s (HSP 60) colocalise in human atheroma and induce tumour necrosis factor-α (TNF-α) and MMP production by MDM (Kol et al., 1998). Both HSP 60 activate human EC, SMC, and MDM, thus contributing to atherogenesis (Kol et al., 1999). Furthermore, MMP may contribute to plaque vulnerability by degrading the ECM of the fibrous cap (Schönbeck et al., 1997).

Immunocompetent cells (i.e. T-cells and MDM) infiltrate atherosclerotic plaques of all stages. Activation of T-cells results in secretion of cytokines such as IFN-γ and TNF-α and -β. Apoptosis of SMC is induced by a combination of IFN-γ and TGF-β (Geng et al., 1998). This is mediated by the

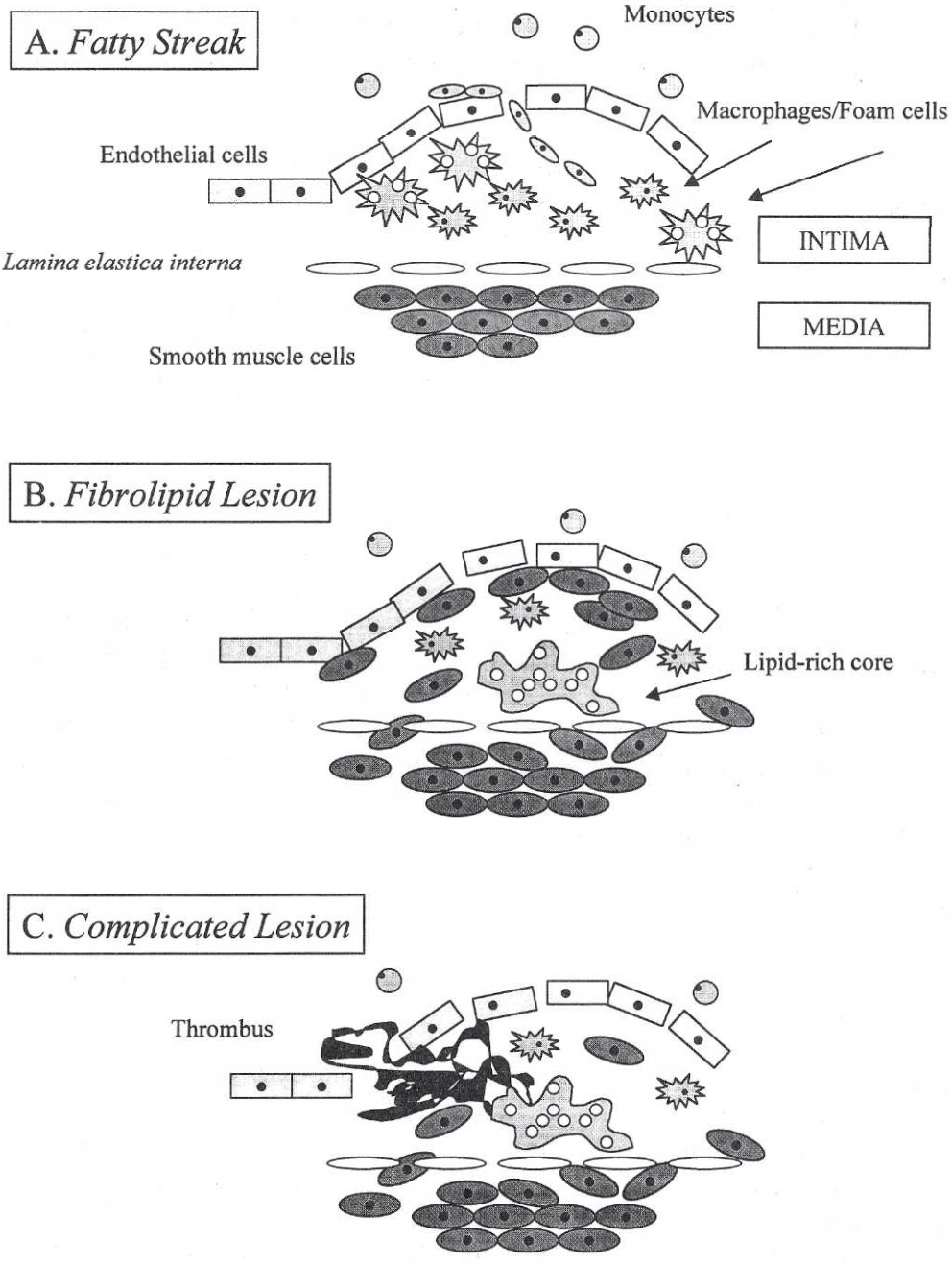

Fig. 2. Developmental stages of atherosclerotic lesions.

generation of gelsolin, a target for the death-promoting cysteinyl-protease caspase-3. Gelsolin-deficient SMC showed resistance to apoptosis induced by these inflammatory cytokines (Geng et al., 1998). Gelsolin may thus contribute to plaque vulnerability.

Although increasing evidence support the involvement of inflammatory and immunological processes in atherosclerosis, principles for cell-cell signalling in atherosclerosis have not been elucidated in detail. A possible mediator of cell-cell communication was discovered recently when it was found that cells within the lesion express elevated levels of the immune mediator CD40 and its ligand CD40L (Mach et al., 1997; Schönbeck et al., 1997). An important role of CD40 in atherogenesis was supported by recent findings in hyperlipidemic apolipoprotein E-deficient mice, in which blocking of CD40 by antibodies reduced their inherited lesion formation (Mach et al., 1998).

Central to the development of the atherosclerotic plaques is the accumulation of LDL, ASMC, MDM, T-cells, and ECM. Initially, it was thought that only SMCs proliferated within atherosclerotic lesions. It is likely, however, that replication of MDM and T-cells may also be important (Rosenfeld and Ross, 1990). Furthermore, both native and oxidised LDL may stimulate the production of PDGF-A chain (see below) transcripts and surface expression of PDGF-Rs in human SMC (Stiko-Rahm et al., 1992). This suggests that LDL may indirectly influence the growth of PDGF-responsive SMCs. This provides a potential link between hypercholesterolemia and activation of SMC growth. Macrophages produce cytokines, including PDGF, which may promote SMC migration from the media to the intima and their subsequent proliferation within the growing lesion.

The involvement of PDGF in intimal hyperplasia has been demonstrated in several *in vivo* experiments (George et al., 1996; Banai et al., 1998). However, it has also been suggested that the proliferative process is initiated by basic fibroblast growth factor (bFGF) and that PDGF is more important for maintaining than initiating the proliferative process by serving as chemotactic attractant and mitogen for SMCs (Reidy et al., 1992). The importance of bFGF in this context was partially confirmed when rat SMC proliferation could be inhibited through the binding of heparin to bFGF (Lindner et al., 1992). *In vivo* data from rat carotid arteries with intimal lesions, showed that reinjury is not controlled by bFGF, suggesting that other factors are important for the second proliferative response (Koyama and Reidy, 1997). Hence, it may be possible that one of these factors is PDGF, which acts as the secondary response after the cells have been "triggered" by FGF.

PDGF ISOFORMS AND THEIR RECEPTORS

PDGF Isoforms

PDGF was first isolated from platelets, hence its name. Today, it is well recognised that this growth factor is produced by a variety of cells. PDGF belongs to a superfamily of its own, which also includes vascular endothelial growth factor (VEGF), a mitogen for endothelial cells (Keck et al., 1989). Recently, a connection between members of the PDGF family was established by Edelberg et al. (1998), who showed that PDGF-AB/PDGF-Rα communication induces expression of VEGF and one of its receptors, which are critical components of normal angiogenesis.

Biologically active PDGF is a homo- or heterodimer of two similar but distinct disulphide-linked A- and B-chains. Hence, mature PDGF occurs as three chimeric isoforms: PDGF-AA, -AB, and –BB (Fig. 3). The PDGF chains have a high amino acid homology of approximately 50%. All isoforms have been isolated from various sources including platelets, vascular SMCs (VSMCs), EC, and macrophages (Stroobant and Waterfield, 1984; Collins et al., 1985; Heldin et al., 1986; Barrett and Benditt, 1988; Hammacher et al., 1988).

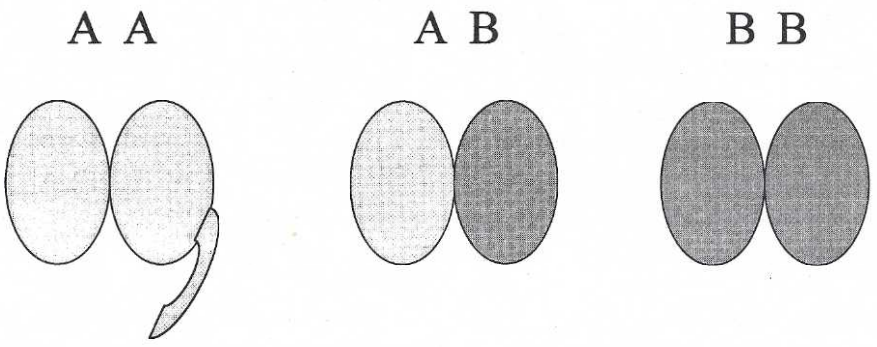

Fig. 3. Naturally occurring PDGF isoforms.

The PDGF-B chain was mapped to the short arm of chromosome 21; region 12.2-13.1 (22q12.2-13.1) (Dalla Favera et al., 1982; Swan et al., 1982) while the human PDGF-A chain has been mapped to the long arm of chromosome 7

(7p) (Stenman et al., 1992). The general structure of these two genes is similar, with seven analogous but differently spaced exons (Bonthron et al., 1988; Rorsman et al., 1988).

PDGF occurs as a cell-retained and a secreted form. Due to alternative splicing, either exon 6 or 7 encodes the carboxy-terminus of the PDGF-A chain (Betsholtz, 1993). The alternative splicing of the PDGF-A chain mRNA results either in a long isoform (PDGF-A_L) containing a basic amino acid sequence corresponding to exon 6 or in a short isoform (PDGF-A_S) lacking this basic C-terminus (Fig. 3). The basic C-terminal tail gives PDGF-A_L cellular and matrix retention properties (Pollock and Richardson, 1992; Andersson et al., 1994) by binding to heparin-like glycosaminoglycans (GAGs) (Fager et al., 1992; Lustig et al., 1996; Lustig et al., 1999). Although the transforming effects of PDGF are independent of this cell retention (LaRochelle et al., 1990), PDGF-A_L may exhibit higher mitogenic activity (Collins et al., 1987) possibly due to increased efficiency of assembly and secretion (Bonthron et al., 1988). It has also been suggested that the basic C-terminal sequence is responsible for targeting a non-secreted form of the PDGF-A chain to the nucleus (Maher et al, 1989).

In contrast, PDGF-B is synthesised only as a long isoform containing an exon 6-encoded basic C-terminal peptide, which has been implicated in membrane translocation (LaRochelle et al., 1991; Östman et al., 1991; Raines and Ross, 1992). However, proteolytic cleavage then removes the basic "tail" after membrane translocation and the short mature PDGF-B isoform is released (Johnsson et al., 1984).

The GAG retention motif of PDGF is conserved in all species sofar investigated, supporting the importance of this sequence. Related VEGF members also have a conserved alternative splicing of exon 6, which further implies a significant biological role for this phenomenon (Keck et al., 1989; Leung et al., 1989; Betsholtz et al., 1990; Maglione et al., 1993; Olofsson et al., 1996).

The *in vivo* importance of the different PDGF chains has been extensively studied in knock-out (KO) mice. Despite the high lethality of homozygous PDGF KO, it has been shown that PDGF-A KO have a severely defective alveogenesis (Boström et al., 1996; Lindahl et al., 1997b), whereas PDGF-B deficient mice demonstrate renal, cardiovascular, and haematological abnormalities with pericyte loss and microaneurysm formation (Levéen et al., 1994; Lindahl et al., 1997a). Thus, PDGF is a key regulator of connective tissue cells also in embryogenesis.

Regulation of PDGF Gene Expression

A general survey of the different signals controlling PDGF expression has been given by Dirks and Bloemers (1996). Despite the role of PDGF in several physiologic and pathophysiologic processes, the molecular mechanisms controlling the transcription of its genes are still poorly understood. The transcription of the PDGF genes seems to be governed by complex interactions of both positive and negative regulatory influences (Kaetzel et al., 1993). The regulatory mechanisms in PDGF production is further discussed in Results and Discussion.

PDGF Receptors

PDGF exerts its mitogenic and chemotactic functions by binding to specific cell surface receptors composed of two subunits; α (PDGF-Rα) and β (PDGF-Rβ). The gene for PDGF-Rβ was cloned in 1988 (Claesson-Welsh et al., 1988; Gronwald et al., 1988) and that of the PDGF-Rα in 1989 (Claesson-Welsh et al., 1989a). This allowed structural comparisons between the two receptor subunits (Claesson-Welsh et al., 1989b).

The PDGF-Rs belong to the receptor tyrosine kinase (RTK) subclass III which belongs to the RTK superfamily. Today, there are five known RTK class III genes; the PDGF-Rα, the PDGF-Rβ, and the receptors for human proto-oncogene c-kit, proto-oncogene fms, and murine flt-3 (Coussens et al., 1986; Yarden et al., 1987; Gronwald et al., 1988; Matsui et al., 1989; Rosnet et al., 1991). Although closely related to the class III subclass, the VEGF receptors (VEGF-Rs) constitute a separate subfamily and form the RTK class V (Rousset et al., 1995; Kondo et al., 1998). Interestingly, the genes for both subfamilies are located in three clusters on different chromosomes. Two of these clusters contains one class V gene and two downstream class III genes. Only one class V gene and one class III gene have been found in the third cluster. The absence of a second gene encoding a class III receptor has arisen speculations about a third, yet unidentified, PDGF-R (André et al., 1992; Rousset et al., 1995).

PDGF isoforms bind to the corresponding PDGF-R subunits in a specific manner (Claesson-Welsh et al., 1989a; Seifert et al., 1989). The extracellular domain of the PDGF-Rα binds both PDGF-A and B-chains with high affinity, whereas the PDGF-Rβ only binds the PDGF-B chain with high affinity (Fig. 4). The high affinity sites for PDGF-AA and –BB in the PDGF-Rα extracellular

domain are probably structurally distinct (Heidaran et al., 1992). Furthermore, specific regions in the PDGF molecule are involved in receptor binding. Thus, mutational analyses have previously shown that loops 1 and 3 of PDGF are important for receptor binding (Clements et al., 1991; Jaumann et al., 1991). Further, it was shown that the loop 2 region is more important for binding to PDGF-Rβ than PDGF-Rα (Andersson et al., 1995).

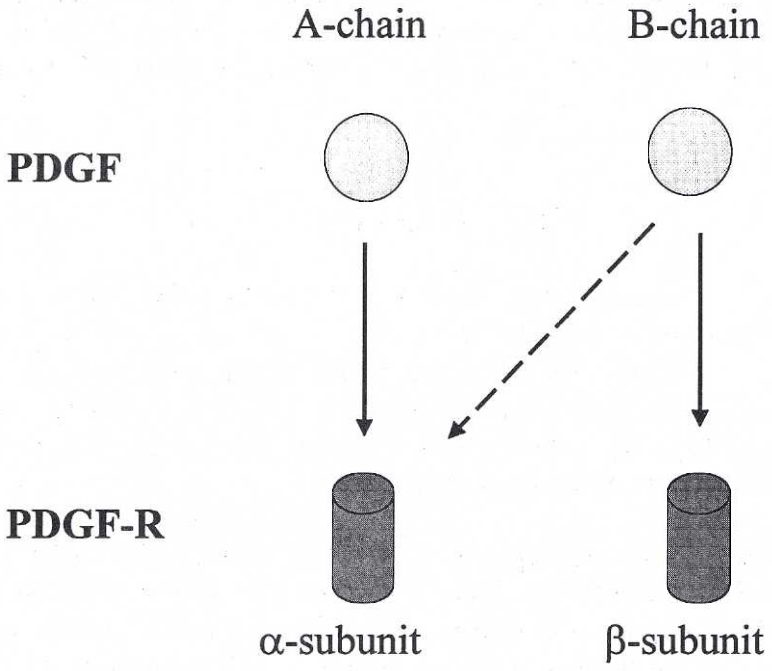

Fig. 4. The PDGF chains and their binding specificity to the corresponding PDGF-R subunits.

The importance of the PDGF-R subunits *in vivo* has been extensively studied through the development of KO mice for both subunits. Homozygous PDGF-Rβ KO die at or shortly after birth and exhibit abnormal kidney development as well as haematological defects similar to PDGF-B KO (Soriano, 1994). PDGF-Rα KO show that this receptor subtype is essential for neural crest development and only heterozygous KO survive (Soriano, 1997). These observations support several of the findings in the corresponding ligand KO and confirm the vital

importance of normal PDGF ligand and receptor interactions also during embryonic development.

Regulation of PDGF Receptor Gene Expression

Knowledge about the regulatory elements involved in the transcription of the PDGF-R genes has been limited so far. Alternative transcripts for both PDGF-Rα and -Rβ have been found (Vu et al., 1989; Afink et al., 1995; Kraft et al., 1996). However, the role of these truncated proteins encoded by alternative PDGF-R transcripts is not known, since none of them have been detected *in vivo*. *Transcriptional regulation* of PDGF-R is described in further detail in Results and Discussion.

PDGF Signalling Through Its Receptors

The PDGF-Rs exist as free transmembraneous monomers in the absence of ligand (Herren et al., 1993). When a dimeric PDGF molecule first binds one subunit and then another, a dimerisation of these PDGF-R subunits occurs (Westermark and Heldin, 1991). It is still not clear if this dimerisation only involves bridging of the two receptor subunits by one dimeric PDGF molecule, or whether the receptor molecules directly engage in dimerisation (Westermark and Heldin, 1991). Dimerisation of receptor subunits by binding to dimeric PDGF is a prerequisite for the mitogenic signal transduction and is coupled to kinase activation and transphosphorylation of receptor molecules (Westermark and Heldin, 1991). There are two possible roles for this dimerisation. Either, kinase activity is triggered by conformational changes, which unfold active sites, or the PDGF-R molecules are brought together, which permits transphosphorylation between the two kinase domains of the subunits through a basal kinase activity (Westermark and Heldin, 1991). Receptor activation results in phosphorylation of tyrosine residues. These phosphotyrosines participate in binding of downstream signal transduction molecules, which recognise specific phosphorylated tyrosines and adjacent amino acid residues (Koch et al., 1991). Most of these sites are outside the kinase domains. These molecules belong to a family of Src homology 2 (SH2) domain molecules (Koch et al., 1991) (Fig. 5).

The binding of PDGF to its receptor elicits an intracellular signalling cascade. Early, it was discovered that PDGF-A and -B chains differ in transforming potential (Beckmann et al., 1988), suggesting that both chains have unique and

specific functions within the cell (Mendoza et al., 1990; Reuterdahl et al., 1993; Inui et al., 1994). It is now clear that different pathways are activated depending on the PDGF isoform (Kondo et al., 1993). Further, the two receptor subunits activate both common and unique signal transduction pathways (Eriksson et al., 1992; Inui et al., 1994). This may explain why different isoforms of PDGF may exert different functions within the target cell.

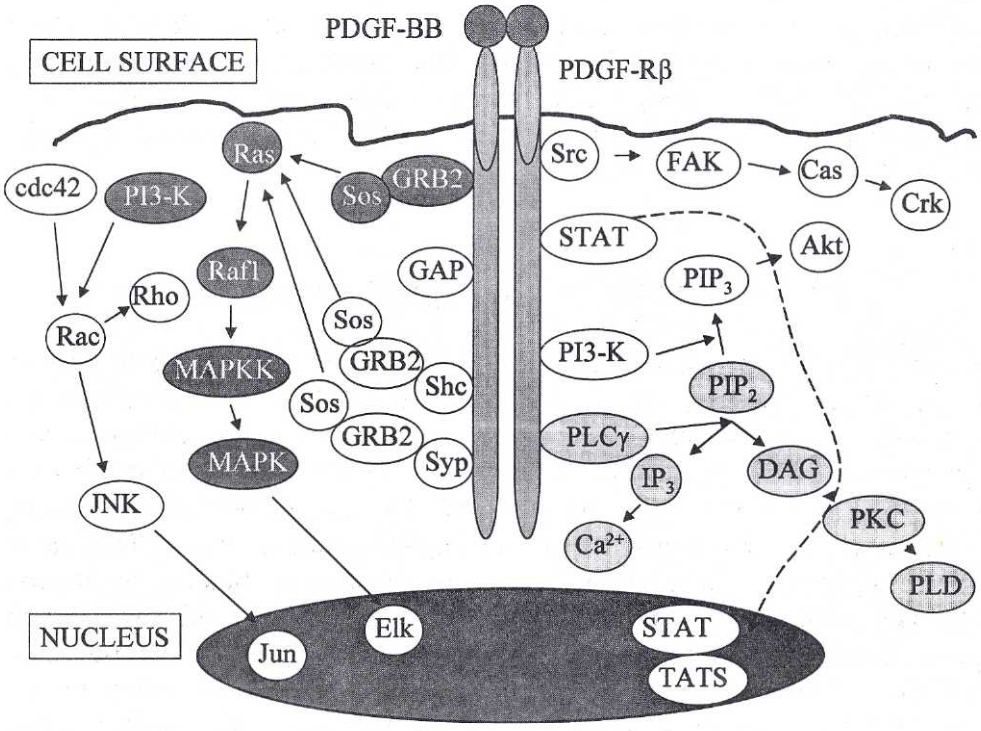

Fig. 5. Simplified model of signal transduction pathways for PDGF-Rβ. *Suggested pathways for proliferation (dark grey) and migration (light grey) are depicted. (Modified from Lindahl (1998)).*

The very rapid and immediate response to PDGF binding includes intracellular Ca^{2+} fluxes and cytoplasmic pH changes (Heldin et al., 1987). Very soon after stimulation, these changes are followed by cytoskeletal rearrangements. The long term responses to PDGF stimulation include migration, proliferation, and differentiation of target cells. Distinct signal transduction pathways are involved when human arterial SMCs migrate or proliferate upon ligand stimulation (Bornfeldt et al., 1995) (Fig. 5).

PDGF-Rs signal through at least five signal transduction pathways, which are all shared with other RTK and additional receptor types (Claesson-Welsh, 1996). PDGF-Rβ has been more extensively studied than PDGF-Rα. Several signal transduction molecules interact with the PDGF-Rβ (Fig. 5). These are signal transduction molecules with enzymatic activity such as phosphatidyl-inositol 3-kinase (PI3-K), phospholipase C-γ (PLC-γ), the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2, the GTPase activating protein of Ras (GAP), as well as several adaptor molecules such as growth factor receptor bound protein (GRB2), Shc, Nck, GRB7, and Crk, and signal transducers and activators of transcription (STATs) (Claesson-Welsh, 1996; Heldin et al., 1998). All signal transduction molecules have different roles in PDGF induced proliferation and migration. A similar detailed information on PDGF-Rα signalling is not available today although a few downstream signalling molecules have been identified (Heldin et al., 1998).

PDGF and SMCs in Atherogenesis

The involvement of PDGF in intimal hyperplasia has been shown by several *in vivo* experiments. Thus, PDGF contributes to neointimal formation after balloon angioplasty in pigs (Banai et al., 1998) and has also an essential role in human SMC migration into the neointima of the saphenous vein (George et al., 1996). Other *in vivo* experiments show that the administration of an anti-PDGF antibody inhibited neointimal SMC accumulation after angioplasty in rats (Ferns et al., 1991). Also, introduction of a gene construct encoding PDGF-B chain into intimal cells of normal porcine iliofemoral arteries resulted *in vivo* in VSMC proliferation and vascular stenosis (Nabel et al., 1993). All these experiments taken together strongly suggest that PDGF is involved in the proliferative process.

PDGF-induced SMC proliferation has a pivotal role in developing atherosclerotic lesions. Although initial proliferation contributes to plaque development and narrowing of the arterial lumen, later this proliferation may contribute to plaque stabilisation by creating a fibrous cap over the lesion. This makes the lesion less prone to rupture. Plaque stability depends on the structural integrity of its ECM skeleton. SMC contribute to this matrix by producing large amounts of collagen and proteoglycans. In fact, collagen and proteoglycan syntheses are increased when SMC change from the contractile to the synthetic state in association with proliferation (Ang et al., 1990; Fager et al., 1995). A role for SMC proliferation in plaque stabilisation is supported by the

observation that disrupted aortic caps contain fewer SMCs and less collagen than intact caps (Wagner et al., 1980; Davies et al., 1993).

Thus, SMC proliferation may have dual effects during lesion development depending on the stage of the lesion. Plaque stability may also be achieved by reducing plasma lipids, which reduces local inflammation and lipid accumulation (Libby and Aikawa, 1998). Instability of the plaque is also a result of macrophage accumulation, activation, and apoptosis of cells (Lee and Libby, 1997). Growth factors may contribute to lesion stabilisation by acting as "survival factors" which prevent cells within lesions from undergoing apoptosis. Thus, PDGF may partially reverse apoptosis in human VSMC and subsequently increase plaque stability (Bennett et al., 1995).

AIMS

The aims of this thesis were to study the *in vitro* regulation of PDGF and PDGF-R expression in the main cell-types represented in the human atherosclerotic lesion. Specifically, I wanted to investigate

- If the expressions of PDGF isoforms and their receptors are affected by changes in cell phenotype
- If this expression is modulated by exogenous cytokines
- If different PDGF homodimers may affect major cellular events, such as proliferation of hASMC and migration of hMDM, which are known to occur in the atherosclerotic lesion

RESULTS AND DISCUSSION

This thesis focuses on the expression of PDGF and its cognate receptors in the main cell types occurring in the atherosclerotic lesion; SMC and MDM. It also provides observations on the effects of phenotypic changes and cytokines on these expressions. Further, it studies PDGF expression during phenotypic changes of EC as well as migration of MDM and proliferation of SMC in response to PDGF. An overview of the different parts constituting this thesis is shown in Fig. 6.

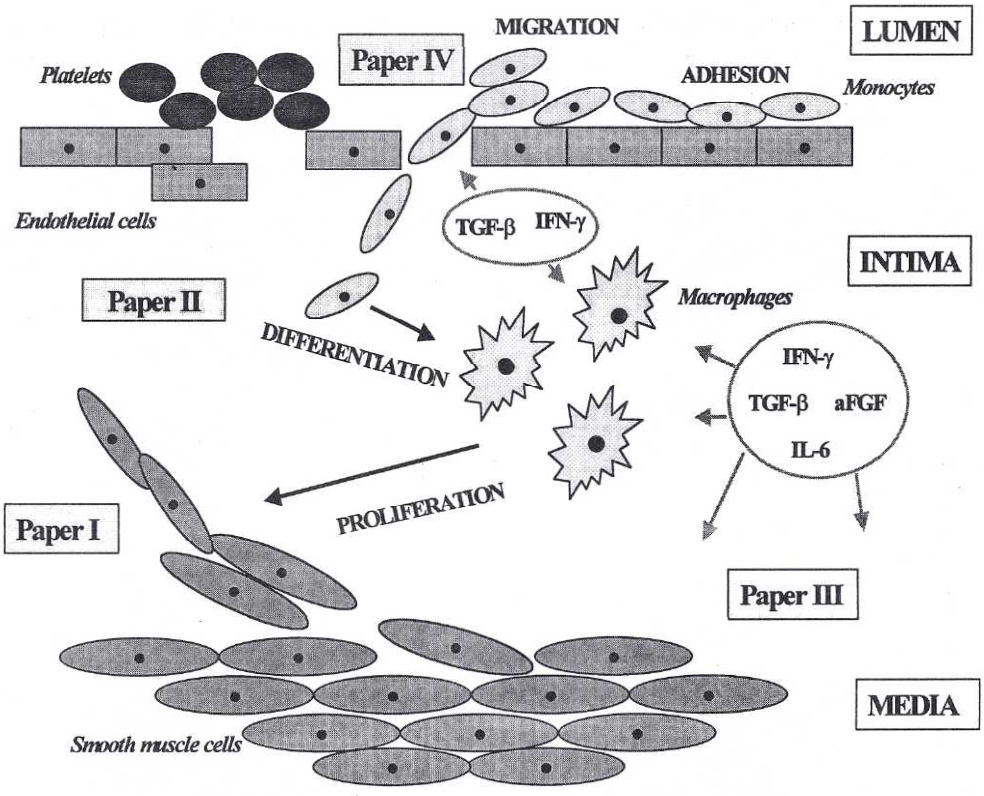

Fig. 6. Schematic representation of the parts of this thesis.

Influence of Phenotype on PDGF and PDGF-R Expression

Human Arterial Smooth Muscle Cells

The accumulation of ASMC in the intima is accompanied by a mitogen-induced change in cell phenotype (Bobik and Campbell, 1993). Growth-stimulated SMCs change their differentiated, contractile phenotype to the dedifferentiated, synthetic state, which is associated with proliferation (Fager et al., 1988; Fager et al., 1989). In the present study, hASMC (positive staining for SMC-specific α-actin) were isolated from the inner media of human uterine arteries (Papers I, II, III). These cells were isolated by explantation and treated as described previously (Fager et al., 1988). Secondary (passage 5 to 8), untransformed, and mycoplasma-free cells were used throughout these studies. Different SMC phenotypes (quiescent, proliferating, and confluent) were established *in vitro* as described elsewhere (Fager et al., 1988; Fager et al., 1989). The different phenotypic states of hASMCs used in Papers I (Figs. 6 and 7) and II (Figs. 6 and 8) were chosen to mimic the *in vivo* situation in developing lesions.

PDGF mRNA Expression

Messenger RNA levels were determined using a quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) as described (Paper II; Wang et al., 1989). At least 100 times higher expression of PDGF-A than PDGF-B mRNA was found in hASMC (Paper II). The PDGF-B mRNA expression was very low in hASMC compared to the other studied cell types.

In the presence of serum, proliferating hASMC grossly decreased total PDGF mRNA levels (Paper II) and both PDGF isoform mRNAs were downregulated in confluent hASMC. Since PDGF-Rβ mRNA was also downregulated in this phenotype (Paper I), these observations suggested that reduced production of endogenous PDGF isoforms and at least one of its receptors may contribute to growth arrest during confluency.

Irrespective of phenotype, only a minor fraction of total PDGF-A mRNA consisted of PDGF-A_L transcripts in hASMC. However, since hASMC are one of the major cell types within the arterial wall, which also expressed high levels of PDGF-A mRNA, the hASMC contribution to the PDGF-A_L pool is probably substantial. A summary of PDGF expression during phenotypic changes of hASMC is given in Table 1.

PDGF-R Expression

A phenotype-dependent expression of PDGF-R mRNAs was previously reported from studies on rat ASMC *in vitro* (Sjölund et al., 1990). The mitogenic stimulation of SMC depends on the concentration of mitogen and the abundance of appropriate receptors.

Quantitative methods were established for the determination of PDGF-R mRNA (quantitative RT-PCR) and protein (quantitative ELISA) levels (Paper I). Our results showed that hASMC from all donors under all studied culture conditions (quiescent, proliferating, and confluent) expressed transcripts for both PDGF-R subunits.

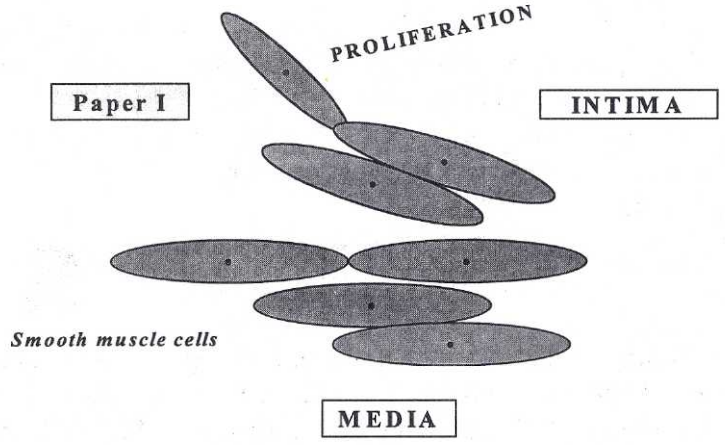

Fig. 7. Schematic representation of Paper I.

The overall PDGF-Rβ mRNA expression was much lower than that of PDGF-Rα. This expression was affected by cell phenotype. PDGF-Rα mRNA levels were constantly high irrespective of phenotype, whereas those of PDGF-Rβ varied with cell phenotype and were thus clearly affected by culture condition. Thus, confluent hASMC, cultured in the presence of serum, tended to downregulate PDGF-Rβ mRNA.

Quiescent hASMC expressed approximately a 13-fold excess of PDGF-Rβ compared to PDGF-Rα protein (Paper I). The relationship between PDGF-Rα and -Rβ protein expressions deduced from the quantitative determinations were indirectly confirmed using immunofluorescent staining on quiescent hASMC

(Fig. 7 in Paper I). The results showed again that the PDGF-Rβ was highly expressed on the surface of quiescent hASMC compared to PDGF-Rα. When sparse cultures were stimulated to proliferate by serum, both PDGF-R proteins were downregulated over time. Although PDGF-Rα mRNA was unaffected by serum, the corresponding protein was highly downregulated. In contrast, PDGF-Rβ mRNA levels decreased upon serum stimulation which was accompanied by some decrease in corresponding PDGF-Rβ protein levels. After 48 hours of serum stimulation, hASMC still showed an approximately 25-fold excess of PDGF-Rβ protein compared to PDGF-Rα. Immunofluorescent staining of confluent hASMC cultures showed that PDGF-Rs were expressed on the surface of these cells even after prolonged serum-stimulation. PDGF-R expression during phenotypic changes of hASMC is summarised in Table 1.

Table 1. Summary of PDGF and PDGF-R expression in hASMC.
Results in SM are shown in relation to cells in SFM.

hASMC Phenotype	**mRNA**	**Protein**
Differentiated and quiescent (SFM)	PDGF-A > PDGF-B PDGF-Rα > PDGF-Rβ	 PDGF-Rα < PDGF-Rβ
Dedifferentiated and proliferating (SM)	PDGF-A - PDGF-A_L - PDGF-B ↓ PDGF-Rα - PDGF-Rβ ↓	 PDGF-Rα ↓ PDGF-Rβ ↓
Confluent and quiescent (SM)	PDGF-A ↓ PDGF-A_L - PDGF-B ↓ PDGF-Rα - PDGF-Rβ ↓	

Hence, our results suggest that the two PDGF-Rs are independently regulated. The discrepancies between PDGF-R mRNA (ratio β/α of 1/100) and protein (ratio β/α of 10/1) levels suggest additional regulatory mechanisms on the transcriptional/post-transcriptional level of PDGF-R gene expression in

hASMC. This is further discussed below. Only PDGF-Rβ mRNA was affected by the phenotypic changes and this subunit was highly expressed on the protein level compared to PDGF-Rα. However, PDGF-Rα protein was more sensitive to serum and was highly downregulated compared to PDGF-Rβ protein upon serum stimulation.

Human Monocyte-Derived Macrophages

When monocytes from the blood enter the intima they start to differentiate into mature macrophages (Fig. 8). MDM were studied during differentiation from monocytes to macrophages in the presence of serum (Paper II) or as early hMDM in the absence of serum (Paper III and IV). Mononuclear cells were isolated from buffy coats by a Ficoll-Hypaque discontinuous gradient (Böyum, 1976) in Papers II, III, and IV. From an aliquot of this cell suspension, monocytes were isolated using magnetic beads on ice (Paper II). This was made to obtain unstimulated monocytes which were neither activated by plating nor serum-stimulation, and to provide the best possible basal value. Hence, Dynabeads M-450/CD14 were used for some isolations. The CD14 antibody specifically isolates monocytes and leaves them attached to the magnetic beads. From these cells, RNA was directly isolated.

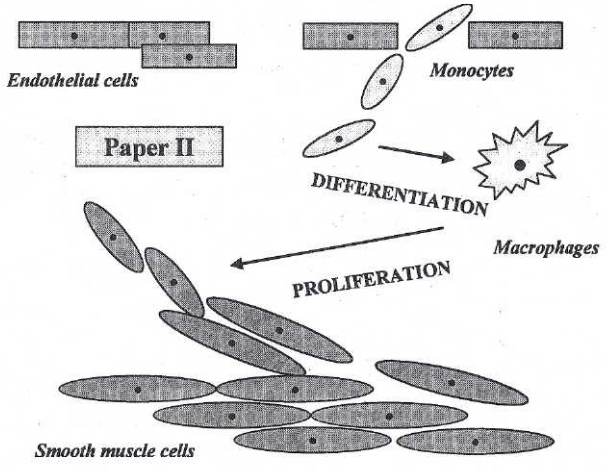

Fig. 8. Schematic representation of Paper II.

PDGF mRNA Expression

The mRNA levels were estimated with quantitative RT-PCR of PDGF isoforms (Paper II; Wang et al., 1989). A major peak in PDGF mRNA expression was seen after 3 days of serum-stimulation. Differentiation of monocytes to macrophages, in the presence of serum, increased both PDGF-A and -B mRNAs. Compared to unplated monocytes, this occurred already after one day in SM. Both PDGF isoform mRNAs were expressed at almost similar levels in hMDM after differentiation for 7 days in SM. This suggested that PDGF production was associated with serum-dependent differentiation of hMDM. This hypothesis was supported by the decreases in PDGF mRNAs in early hMDM which were maintained in SFM (Paper III).

The differentiation of monocytes to macrophages increased the transcription of PDGF-A_L from 10% to 40% of total PDGF-A.

Table 2. Summary of PDGF and PDGF-R expression in MDM.
Results for dedifferentiated early hMDM are shown as changes in PDGF/PDGF-R mRNA expression over time in culture. In differentiated hMDM, results for PDGF mRNA are shown in relation to the expression in unplated, dedifferentiated monocytes (Day 0 in Paper II) whereas PDGF-R expression is shown as changes over time in culture.

hMDM Phenotype	**mRNA**	**Protein**
Dedifferentiated cells early hMDM (SFM)	PDGF-A ↓ PDGF-A_L ↑ PDGF-B ↓ PDGF-Rα > PDGF-Rβ	 PDGF-Rα < PDGF-Rβ
Differentiated cells hMDM (SM)	PDGF-A ↑ PDGF-A_L ↑ PDGF-B ↑ PDGF-Rα↑ PDGF-Rβ -	

PDGF-R Expression

Semi-quantitative RT-PCR of PDGF-R mRNA (Paper IV) showed that hMDM, in response to serum, upregulated PDGF-Rα mRNA during differentiation. A significant continuous increase compared to Day 1 was observed ($P>0.002$). In contrast, PDGF-Rβ mRNA was not affected by hMDM differentiation and was expressed at a constant level during 7 days of culturing ($P>0.4$) (Fig. 9; unpublished results).

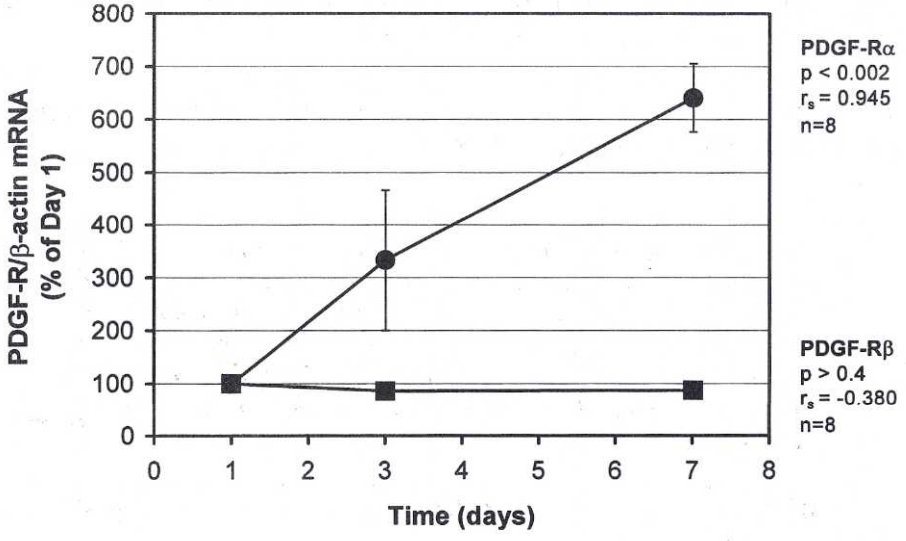

Fig. 9. PDGF-R mRNA expression in differentiating hMDM. *PDGF-R expression in each RNA sample was related to the expression of the house-keeping gene β-actin. Results are shown as the mean of three donors, each run in triplicate. r_s denotes Spearman's rank coefficient.*

In early hMDM (plated monocytes cultured in SFM), PDGF-Rα mRNA was expressed at much higher levels than PDGF-Rβ mRNA, as judged from semi-quantitative RT-PCR analyses. In contrast, quantitative ELISA showed that PDGF-Rα protein levels were much lower than the corresponding levels of PDGF-Rβ (Paper IV, Figs. 6 and 11). Generally, PDGF-Rα was expressed at high mRNA and low protein levels. These results were similar in hASMC (Paper I). Again, this suggested a complex transcriptional/post-transcriptional regulation of PDGF-Rs. Either may PDGF-Rα mRNAs be only partly processed to competent cell surface receptors, or cell surface receptors

efficiently internalised after binding endogenously produced PDGF by hMDM (Paper II). Such internalised receptor-ligand complexes would not have been recognised by the ELISA used in Papers I and IV. A summary of PDGF and PDGF-R expression in hMDM is given in Table 2.

Human Adult Vein Endothelial Cells

In case of an injury to the endothelium, EC start to proliferate and move into the damaged area. Proliferation is associated with dedifferentiation of hASMC and EC. Human adult vein EC (hAVEC) (positive staining for von Willebrand Factor) were isolated from stripped varicose veins. Cells in passage 4 were cultured and studied in different phenotypes (proliferating and confluent). Human AVEC were used instead of arterial EC due to the difficulty in obtaining large amounts of human arterial EC.

PDGF mRNA Expression

Quantitative RT-PCR analyses showed that hAVEC expressed higher levels of PDGF mRNA compared to both hASMC and hMDM (Paper II, Figs. 6 and 8). Both PDGF isoforms were expressed at similar levels. In the presence of serum, proliferating hAVEC grossly increased total PDGF mRNA levels. Confluent hAVEC, still in the presence of serum, tended to downregulate transcripts for PDGF-A.

Irrespective of phenotype, hAVEC produced 40% of total PDGF-A transcripts as PDGF-A_L mRNA. A summary of PDGF expression in hAVEC during phenotypic change is given in Table 3.

Table 3. Summary of PDGF expression in hAVEC. *Results are shown as a comparison between the two phenotypes.*

hAVEC Phenotype	**mRNA**
Dedifferentiated and proliferating (SM)	PDGF-A ↑ PDGF-A_L – PDGF-B ↓
Confluent and quiescent (SM)	PDGF-A ↓ PDGF-A_L – PDGF-B ↑

In summary, changes in cell phenotype modulated the expression of PDGF and PDGF-Rs in different cell types. Differences in estimated mRNA and protein levels for PDGF-Rs suggested that the expressions of these genes are governed by complex transcriptional/post-transcriptional mechanisms.

In ASMC, serum induced dedifferentiation of cells. Thus, the presence of serum in proliferating (dedifferentiated) cultures of hASMC reduced PDGF–B mRNA as well as PDGF-R, compared to quiescent cells. This suggests that growth arrest in these cells could partially be caused by downregulation of PDGF-B and PDGF-Rs.

In hMDM, serum stimulation resulted in cell differentiation. During this process, both PDGF-A and -B mRNAs were upregulated compared to unplated monocytes. Also the proportions of PDGF-A_L mRNA were increased during differentiation. PDGF-Rα but not PDGF-Rβ mRNA was upregulated by serum in hMDM. This raises possibilities of a physiological importance of these findings *in vivo*, and suggests that PDGF might be important during macrophage recruitment and differentiation in the intima. Our results support a specific role of PDGF isoforms in monocyte migration (see Migration of Early hMDM below).

EC expressed highest PDGF mRNA levels compared to hASMC and hMDM. These cells also had the highest expression of PDGF-A_L which constituted 40% of total PDGF-A mRNA.

Influence of Cytokines on PDGF and PDGF-R Expression

The cytokines in these studies were chosen on the basis of the criterion that they are produced by cells within the atherosclerotic lesion. Thus, aFGF, IL-6, and TGF-β are produced by both hMDM (Rappolee and Werb, 1992; Brogi et al., 1993) and SMCs (Loppnow and Libby, 1990; Agrotis et al., 1994; Chotani et al., 1995), whereas IFN-γ is secreted from activated T-cells in the lesions. Acidic FGF induces angiogenesis *in vivo*, is involved in differentiation of a variety of cells, and is a chemoattractant and mitogenic stimuli for EC *in vitro* (Vainikka et al., 1994). IL-6 is a pleiotropic cytokine which may both promote and inhibit cell growth and induce differentiation in a variety of cells (Narazaki and Kishimoto, 1994). TGF-β has been implicated in various biological processes such as: growth and differentiation of cells, regulation of ECM protein production, an important role in the function and differentiation of the immune system, wound healing and tissue repair, as well as an important

role in embryogenesis (Gitelman and Derynck, 1994). IFN-γ is responsible not only for macrophage activation, but also acts as an antiviral agent, induces the expression of many key molecules (class I and class II MHC antigens, nitric oxide synthase, and cytokines such as IL-1), and has been implicated to participate in the development of autoimmune diseases (Gray, 1994).

All cytokine incubations were made under serum-free conditions in order to avoid interference from serum factors in the experiments. The viability of plated monocytes cultured for three days in SFM and SM was high and comparable between the media when checked daily by trypan blue exclusion and release of lactate dehydrogenase.

Table 4. Influence of exogenous cytokines on PDGF expression in dedifferentiated hASMC in SFM.
Results are shown as the expression in cytokine-stimulated cells compared to controls without cytokine stimulation.

hASMC + Cytokines	mRNA	Protein
IFN-γ	PDGF-A – PDGF-A_L ↑ PDGF-B -	PDGF-AB -
TGF-β	PDGF-A ↑ PDGF-A_L ↑ PDGF-B -	PDGF-AB ↑
aFGF	PDGF-A ↑ PDGF-A_L - PDGF-B -	PDGF-AB -
IL-6	PDGF-A - PDGF-A_L – PDGF-B ↑	PDGF-AB ↑

Human Arterial Smooth Muscle Cells

PDGF Expression

Messenger RNA levels were determined with a quantitative RT-PCR method (Paper II; Wang et al., 1989) and PDGF-AB protein levels with a commercially available PDGF-AB protein ELISA from R&D Systems. In differentiated hASMC, PDGF-A mRNA was increased by TGF-β and aFGF whereas PDGF-B

mRNA was slightly increased by IL-6. Both TGF-β and IL-6 increased PDGF-AB protein secretion (Paper III, Figs. 6 and 10). Increased proportions of PDGF-A_L mRNA were found in hASMC after IFN-γ stimulation. The findings in hASMC were consistent with previous findings in EC where IFN-γ upregulated PDGF-A_L mRNA (Zhao et al., 1995). Although the relative increase in PDGF-A_L mRNA in hASMC was small, the simultaneous increase in total PDGF-A in response to TGF-β suggests that the absolute upregulation of PDGF-A_L at the transcriptional level may be substantial in these cells. A summary of PDGF expression in cytokine-stimulated hASMC is given in Fig. 4.

Early Human Monocyte-Derived Macrophages

PDGF Expression

PDGF mRNA and PDGF-AB protein levels were determined as described above for hASMCs. In early hMDM in SFM, PDGF-A mRNA was increased by IFN-γ and PDGF-B mRNA by both IFN-γ and TGF-β (Paper III, Figs. 6 and 10). Only TGF-β increased PDGF-AB protein secretion significantly. Increased proportions of PDGF-A_L mRNA were found only after TGF-β stimulation. It has been shown previously that TGF-β specifically upregulates PDGF-A_L mRNA in EC (Zhao et al., 1995).

Previous reports show that IFN-γ increases PDGF-B mRNA (Wangoo et al., 1993) and PDGF-BB protein (Badgett et al., 1996) in alveolar macrophages or downregulates both PDGF-A and –B mRNAs in hMDM (Kosaka et al., 1992). The discrepancy between our results in early hMDM and those of Kosaka et al. (1992) in MDM probably depends on differences in culture systems and phenotypic state of the cells. This possibility is supported by the observation that IFN-γ has no effect on any PDGF isoform mRNA in transformed monocytic THP-1 cells (Nagashima et al., 1994).

PDGF-R Expression

Using a quantitative ELISA for PDGF-Rs as described in Paper I, we found that early hMDM increased both PDGF-Rα mRNA and protein upon IFN-γ stimulation. TGF-β had only minor effects on both PDGF-R subunits (Paper IV, Figs. 6 and 11). A summary of PDGF and PDGF-R expression in cytokine-stimulated early hMDM is given in Table 5.

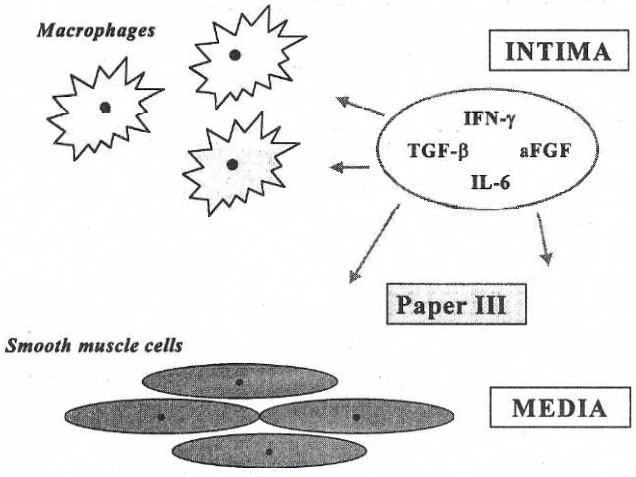

Fig. 10. Schematic representation of Paper III.

In summary, the cytokine studies showed that the cytokines act in a cell type-specific manner. Thus in hMDM, IFN-γ-stimulation upregulated both PDGF-A and –B mRNAs, and also showed major effects on PDGF-R expression in these cells. TGF-β only increased PDGF-B mRNAs but affected the proportions of PDGF-A_L mRNAs which increased after TGF-β stimulation. PDGF-AB protein secretion was induced by TGF-β.

In hASMC, PDGF-A mRNA levels were increased after stimulation with TGF-β and aFGF. Increased levels of PDGF-A_L were induced by both IFN-γ and TGF-β. Only IL-6 upregulated PDGF-B mRNA expression in hASMC.

Interestingly, apoptosis, which is a feature in advanced atherosclerotic lesions, is induced in VSMC by a combination of IFN-γ and TGF-β (Geng et al., 1998). Although this is mediated through the generation of gelsolin (c.f. Development of Atherosclerosis) one might speculate that the increase in PDGF could act on these cells as a "survival factor" thus rescuing them from undergoing apoptosis and thereby contribute to plaque stability. Thus, the balance between exogenous cytokines and PDGF expression may determine plaque stability. It has been shown that IFN-γ alone cannot induce VSMC apoptosis but it induces cell death after simultaneous treatment with TNF-α and/or IL-1β (Geng et al., 1996). *In vivo*, several cytokines act simultaneously on target cells.

Table 5. Influence of exogenous cytokines on PDGF expression in early hMDM.
Results are expressed compared to controls in SFM.

hMDM + Cytokines	mRNA	Protein
IFN-γ	PDGF-A ↑ PDGF-A_L - PDGF-B ↑ PDGF-Rα ↑ PDGF-Rβ -	PDGF-AB - PDGF-Rα ↑ PDGF-Rβ ↓
TGF-β	PDGF-A_L ↑ PDGF-B ↑ PDGF-R minor effects	PDGF-AB ↑ PDGF-R minor effects
aFGF	PDGF -	PDGF-AB N.D.
IL-6	PDGF -	PDGF-AB N.D.

Regulation of PDGF and PDGF-R Expression

Regulation of PDGF Expression

The results obtained in Papers II and III showed that the expression of PDGF-A and -B genes was independently regulated and depended on cell type, cell phenotype, and exogenous influences.

Hence, the effects of cytokines varied with cell type in our studies. A cell type-specific cytokine effect has also been reported by others (Gay and Winkles, 1990; Winkles and Gay, 1991; Kosaka et al., 1992; Wangoo et al., 1993). It has been shown that TGF-β increases PDGF-A mRNA levels in human fibroblasts (Paulsson et al., 1988) and VSMC (Majack et al., 1990), while it stimulates PDGF-B mRNA expression (Leof et al., 1986) or the expression of both isoforms (Kavanaugh et al., 1988) in other cells. IL-6 increases PDGF-A and –B chains in human umbilical vein EC (Gay and Winkles, 1990) or has no effect on PDGF-A transcripts in human saphenous vein SMCs (Winkles and Gay, 1991) or on PDGF-AB protein in astrocyte-enriched cultures (Ceccherini Silberstein et al., 1996).

Independently regulated expression of PDGF-A and –B transcripts in other cell types has been reported previously (Goustin et al., 1985; Heldin et al., 1987;

Paulsson et al., 1987; Westermark and Heldin, 1991). Thus, while many tumour cell lines express either one or both of the PDGF isoform mRNAs (Heldin et al., 1987; Westermark and Heldin, 1991) the PDGF-A mRNA seems to be predominantly expressed, for example, in skeletal myoblasts and fibroblasts (Sejersen et al., 1986; Paulsson et al., 1987) and PDGF-B mRNA in placental cytotrophoblasts (Goustin et al., 1985).

Transcriptional regulation occurs due to 5'untranslated GC-rich sequences (UTS) which may influence gene expression by being potential sites for transcriptional regulatory activity (Bonthron et al., 1988). Such regions in the promoter of the PDGF-A gene have been shown to contain mechanical strain-responsive elements to which the transcription factors early growth response gene product (Egr-1) and possibly Sp-1 binds (Wilson et al., 1998).

The effects of shear stress on PDGF expression is interesting, since cells within the vasculature are constantly exposed to fluid mechanical forces generated by the pulsatory blood flow. Monocytes are subjected to such varying shear stress in the blood stream, at branch points of arteries due to turbulent blood flow and at sites of arterial narrowing due to increased flow velocity. Furthermore, atherosclerotic plaques preferentially occur at sites with turbulent flow and increased shear stress. Both PDGF-A and –B mRNA levels are strongly induced in response to shear stress in human umbilical vein EC (Hsieh et al., 1991). This is mediated by fluid-shear-stress responsive elements (SSRE) in the promoters for both PDGF-A and PDGF-B genes, which bind Egr-1 (Khachigian et al., 1996; Khachigian et al., 1997). It is now recognised that the induction of Egr-1 is a common theme in vascular injury (Khachigian and Collins, 1997).

Another stress-related influence on the cells within the vessel wall are the varying levels of oxygen concentration. Hypoxia results in both vasoconstriction and vascular remodelling (Leach and Treacher, 1995). These processes result in a narrowing of the blood vessel and reduced blood flow. During chronic hypoxia, SMC start to proliferate and ECM accumulates rapidly. This results in vessel wall remodelling. Oxygen is a potent regulator of PDGF-B gene transcription in human umbilical vein EC (Kourembanas et al., 1990).

Further, it has been shown that the PDGF-A promoter region has potential for bi-directional activity (i.e. transcription of the gene can occur in both directions) which may regulate transcription. The bi-directional activity is influenced by 5′-UTR and the serum concentration in the culture medium (Takimoto and Kuramoto, 1994). It was later confirmed that serum regulates PDGF-A gene expression at the transcriptional level (Takimoto and Kuramoto, 1995).

Post-transcriptional regulation of gene expression is achieved through affecting mRNA stability and/or splicing of primary transcripts (Betsholtz, 1993). Messenger RNA degradation in eukaryotic cells is a common theme in the regulation of gene expression. Messenger RNA instability is determined by several elements, including the presence of AU-rich 3'-sequences (Sachs, 1993). Both the PDGF-A and –B chain mRNA contain such AU-sequences in their 3'-UTS with putatively destabilising functions (Betsholtz, 1993). Furthermore, the 5'-UTS of the PDGF-B mRNA are involved in determining degradative pathways for alternate mRNAs (Fen and Daniel, 1991).

Translational regulation by a reversible binding of RNA/protein interaction in the 5'-UTS of the mRNA may be a general theme for regulating gene expression (Melefors and Hentze, 1993). It has been shown for PDGF-B mRNA that 5'-UTS are involved in inhibiting translation (Ratner et al., 1987; Rao et al., 1988).

In our studies, the changes in PDGF mRNA levels did not always correlate with protein secretion in a very consistent way (Paper III). This could be due to a short half-life of the mRNA, which may reduce protein translation. Since the applied ELISA only determines heterodimeric PDGF-AB protein, the secretion of homodimeric PDGF-AA and -BB protein will not be detected. It is not known whether dimerisation of PDGF-A and/or -B monomers occur at random or if it is regulated. For example, increased levels of PDGF-A mRNA might lead to preferential formation of PDGF-AA and this will not be detected by the PDGF-AB ELISA. Furthermore, receptor-ligand internalisation due to autocrine stimulation could reduce PDGF protein in the cell culture supernatants. Finally, the applied ELISA only determines secreted PDGF-AB, thus cell-associated PDGF-AB is not detected with this assay.

Regulation of PDGF-R Expression

Transcriptional regulation has been shown to occur for PDGF-Rs. Thus, the PDGF-Rα gene contains at least two promoters in its genomic sequence, which are responsible for different transcripts (Afink et al., 1995; Kraft et al., 1996). Developmentally regulated PDGF-Rα transcripts lacking exon 14 have also been reported (Mosselman et al., 1994). A similar pattern was observed for PDGF-Rβ where different forms of transcripts for this subunit are produced, either due to alternative usage of two transcriptional promoters or to post-transcriptional processing (Vu et al., 1989).

One possible explanation for the discrepancies between PDGF-R mRNA and protein levels in Papers I and IV is the occurrence of PDGF-Rβ mRNAs and proteins truncated in their intracellular domains. Such truncated forms could have been detected with the antibodies used in our ELISA but not with the RT-PCR primers used for determining mRNA levels. However, sofar only PDGF-R β mRNAs with extracellular domain deletions have been detected (Vu et al., 1989). This provides another explanation to our results, if similar truncated forms of PDGF-Rα transcripts occurred. Such species would not have been detected by our ELISA antibodies. RT-PCR experiments were thus performed with several other primer pairs located in different regions of the PDGF-Rα sequence (Paper I). Our findings were indirectly compatible with the possibility of alternative promoter usage during transcription of the PDGF-Rα gene in hASMC resulting in PDGF-Rα with truncated extracellular domains (Paper I).

The results in Papers III and IV show that several cytokines affect both PDGF-R and PDGF expression. The finding that IFN-γ upregulated both PDGF-A and PDGF-Rα in early hMDM suggests that this cytokine may have potential importance during early hMDM recruitment to sites of injury and inflammation. This results in increased PDGF expression and enhanced responsiveness to PDGF by early hMDM.

The mechanism behind the stimulatory effect of IFN-γ in early hMDM is unclear. It was found previously that the action of IFN-γ is mediated through the Jak/STAT pathway and phosphorylation of STAT1 leads to dimerisation of STAT proteins and their translocation to the nucleus (Fig. 5). Here, the STAT1 complexes bind to IFN-γ Activated Sequences (GAS), which leads to transcriptional activation of the target gene (Brown, 1998). The inability of STAT1 KO mice to respond to IFN-stimuli, confirms that STAT1 has a crucial and obligate role in IFN-γ signalling (Durbin et al., 1996; Meraz et al., 1996). Interestingly, both PDGF-Rα and PDGF-A contain STAT-binding sites in their promoter regions, suggesting that this could be a possible mechanism of the IFN-γ action in early hMDM. Further, it was previously shown that PDGF-BB directly activates the transcription factor STAT1 (Yamamoto et al., 1996), and that PDGF-BB through the interaction with PDGF-Rβ phosphorylates multiple Jak family kinases and STAT proteins (Vignais et al., 1996). Finally, IFN-γ also potentiates the DNA-binding activity of STAT1 in response to PDGF, resulting in increased DNA synthesis (Marra et al., 1996).

In TGF-β-stimulated hASMC, both PDGF-A mRNA and PDGF-AB protein were increased. Human MDM only upregulated PDGF-B mRNA in response to this cytokine. The mechanisms by which TGF-β influences gene expression are

not well understood. It has been shown that the interaction of TGF-β with its receptor phosphorylates the intracellular signal transduction molecule Smad2 (Eppert et al., 1996; Souchelnytskyi et al., 1997). Phosphorylated Smads form heteroduplexes which translocate to the nucleus were they interact with specific DNA sequences thus resulting in gene transcription. Since little is known about TGF-β-signalling sofar, it can not be excluded that the signalling through Smad proteins is not obligate and that other mechanism may be involved when TGF-β exerts its cellular effects.

Biological Implications of PDGF and PDGF-R Expression

Proliferation of hASMC

It has been suggested that the responsiveness of cells to PDGF stimulation is regulated by the relative abundance of PDGF-Rs on the cell surface. Since proliferation rather than migration of SMCs plays a central role during the development of atherosclerotic lesions, we focused on the proliferative rather than the migratory response of hASMC to PDGF stimulation. Therefore, the responsiveness of hASMC to stimulation with different exogenous recombinant PDGF homodimers was studied (Paper I). Incorporation of thymidine into DNA was used to determine the proliferative effect of the different exogenously added PDGF homodimers.

The proliferation of hASMC in response to different PDGF homodimers (PDGF-AA_L, PDGF-AA_S, and PDGF-BB_S) showed a bimodal dose-dependence with increasing thymidine indices up to a maximum stimulation at about 5 nmol/L of homodimer. A small but consistent dose-dependent decrease in these indices was observed above this concentration. Similar bimodal responses have been shown for bFGF-induced proliferation (Bono et al., 1997) and for PDGF-induced migration (Koyama et al., 1994). A possible explanation for these observations is that the dimerisation of PDGF-R subunits required for optimal signal transduction is maximal at a molar ratio of 1:2 and that above this level, additional ligand will block signalling by monosaturating the PDGF-R subunits.

Different exogenous PDGF homodimers induced rather similar proliferative responses in hASMC in spite of the 10-fold difference in abundance of PDGF-Rα and PDGF-Rβ proteins. The fact that the more abundant PDGF-Rβ only responds to PDGF-B and that PDGF-A dimers were almost as efficient stimulators of DNA synthesis, may suggest that signalling through the PDGF-Rα is more important for the proliferative response in hASMC. The similar

capacity of different PDGF homodimers to induce proliferation in hASMC suggested that the number of PDGF-Rs on the surface of these cells were not the only determinants for maximal cell stimulation.

Prolonged SMC proliferation, as observed after reconstructive vascular surgery, could be achieved by the release of long PDGF isoforms from the ECM. Although the results in Paper II suggested that SMC express low concentrations of PDGF-A_L, their total contribution to the PDGF-A_L pool may be substantial due to the large number of these cells in the arterial wall. The comparatively high expression of PDGF-A_L in hAVEC and its upregulation during monocyte differentiation may contribute to a depot of PDGF-A_L in the arterial wall. The basic C-terminal extensions in the long PDGF isoforms may bind to GAGs in the surrounding matrix (Lustig et al., 1996; Lustig et al., 1999). This bound PDGF-A_L can be released from its depot through the action of proteases such as coagulation factor Xa which has been shown in rat VSMCs (Ko et al., 1996). Recent data confirm that factor Xa by binding to high affinity sites exerts a mitogenic effect on VSMCs via the release of PDGF, which subsequently leads to the activation of MAP kinase, DNA synthesis, and cell growth (Herbert et al., 1998). Dissociation of bound long PDGF isoforms may then induce cell proliferation through paracrine (cells stimulating neighbouring cells) or autocrine (cells stimulating themselves) mechanisms long after the initial PDGF secretion. In fact, PDGF-A_L is upregulated *in vivo* during active wound healing in response to PDGF-BB treatment (Pierce et al., 1995) and also present in cardiac allografts but not normal hearts (Zhao et al., 1995).

Furthermore, the increase in PDGF-B mRNA induced by some of the cytokines (Paper III), may result in increased production of PDGF-BB_L, which is translocated over the cell membrane, bound to GAGs, and then proteolytically cleaved and released as short isoforms (Johnsson et al., 1984). Thus, PDGF-induced cell proliferation and migration could be sustained by the accumulation of long PDGF isoforms bound on GAGs in the arterial wall (Andersson et al., 1994). Interestingly, the cytokines studied in Paper III can also be stored in the cellular matrix (López-Casillas et al., 1991; Ramsden and Rider, 1992; Nagashima et al., 1994; Camejo et al., 1995; Lortat-Jacob et al., 1995) and their subsequent release may also result in prolonged effects on hASMC proliferation by inducing PDGF expression, leading to intimal hyperplasia long after the initial injury.

Migration of Early hMDM

The accumulation of monocytes at the sites of inflammation and endothelial injury is regarded as an early step in atherosclerosis (Holvoet and Collen, 1997). Since we have shown that early hMDM express both PDGF and PDGF-Rs, the question arouse whether PDGF could contribute to early hMDM migration. Paper IV investigated the potential role of PDGF in this process in early hMDM. For migration analyses, PDGF homodimers were used at a concentration of 10 nM in the upper and/or the lower compartment in a modified Boyden-Chamber. This concentration was chosen since we previously found maximal induction of hASMC proliferation between 5-10 nM of exogenous PDGF (Paper I).

Two different migratory responses are seen in PDGF-stimulated cells; either stimulated random motility (chemokinesis) or directed migration (chemotaxis) towards increasing concentrations of PDGF (Grotendorst et al., 1982). Chemotaxis is of physiological relevance *in vivo* during wound healing when fibroblasts, monocytes, and macrophages migrate to sites of injury.

Whereas PDGF-induced DNA synthesis and cell proliferation involves activation of the *Ras* proto-oncogene, mitogen-activating protein (MAP) kinase kinase, and MAP kinase, the migration of cells is caused by the activation of the diacylglycerol-pathway (phosphatidyl-inositol turnover) and an elevation of intracellular calcium levels (Fig. 5). This leads to disassembly of actin filaments and enables the cell to protrude filopodia (Bornfeldt et al., 1995). Previous studies show that activation of the PDGF-Rβ leads to both proliferation and migration (Bornfeldt et al., 1995). Although PDGF-Rα also transduces a mitogenic signal, it mediates migration in a cell-specific manner and stimulation of PDGF-Rα may even inhibit migration (Heldin et al., 1998). Inhibition of the migratory response has been attributed to autophosphorylation of certain tyrosine residues in the PDGF-Rα (Yokote et al., 1996).

Early hMDM exhibited basal motility even in the absence of exogenous PDGF (Paper IV). This may be due to auto- or paracrine stimulation from endogenous PDGF (Paper II and III) or other factors. Only exogenous PDGF-BB_L stimulated motility of early hMDM across the filters whereas neither PDGF-AA_L, PDGF-AA_S, nor –BB_S did (Paper IV). As could be concluded from simple checkerboard experiments, this response was rather an increase in random motility (chemokinesis) than directed migration (chemotaxis). IFN-γ stimulation (known to activate macrophages) upregulated PDGF-Rα which further resulted in increased directed migration (chemotaxis) towards PDGF-AA_L. Interestingly,

IFN-γ induced both PDGF (Paper III) and PDGF-R expression (Paper IV) in early hMDM. TGF-β had only minor effects both on PDGF-R expression and on PDGF-induced motility of early hMDM.

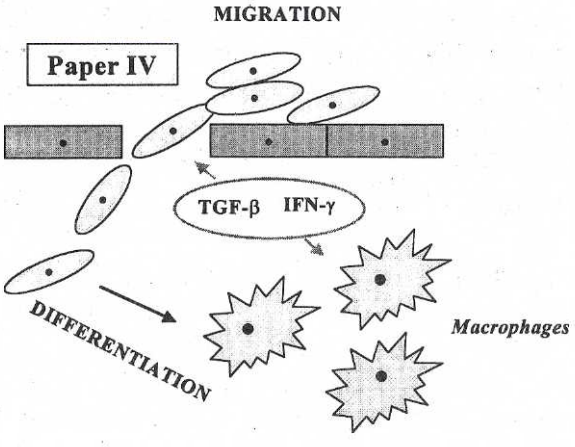

Fig. 11. Schematic representation of Paper IV.

These results suggest that PDGF-AA_L may be involved in attracting activated monocytes to sites of injury. This effect of PDGF-AA_L is mediated through an upregulation of the PDGF-Rα expression and might suggest an additional role of PDGF-AA_L in inflammation and tissue repair

CONCLUDING REMARKS

The results in this thesis indicate that several cell types occurring in atherosclerotic lesions express PDGF and PDGF-Rs *in vitro*. This expression can be modulated by phenotypic changes and cytokines. Increasing levels of PDGF stimulates the proliferation of SMCs and may direct hMDM to sites of a growing atherosclerotic lesion. The effect of PDGF is further sustained by the occurrence and upregulation of long PDGF isoforms, which bind to matrix components. The results suggest that PDGF is a pivotal molecule for cell accumulation in atherosclerotic lesions, not only as a mitogen for hASMC but also as a chemoattractant for early hMDM.

FUTURE PERSPECTIVES

Increasing knowledge about the expression and function of PDGF isoforms and their receptors may extend the possibility to control cell accumulation and migration in the arterial wall. Recently, it was shown that lipid lowering reduced levels of PDGF-B in the arterial intima (Aikawa et al., 1998), suggesting an indirect strategy for reducing PDGF-induced proliferation. A direct approach for reducing PDGF-induced cell accumulation could be to intervene with the PDGF/PDGF-R expression or the signalling pathways activated by the binding of PDGF to its receptors.

Since other factors, such as bFGF, have been implicated in SMC proliferation, it is interesting to pin-point the exact significance of PDGF for the processes of proliferation and migration. This can be elucidated by reducing/inhibiting PDGF-R or PDGF expression in the arterial wall through preventing transcription of these genes by antisense oligonucleotides. Oligonucleotide-based gene therapy has been used for treatment of diseases were no effective therapy presently exists. Now it is also emerging as a potential strategy for treatment of cardiovascular diseases (Morishita et al., 1998).

The increasing levels of PDGF-Rα after IFN-γ stimulation and its potential importance for hMDM migration suggests that this regulatory pathway may be of potential importance. Increasing the knowledge of the transcription factors and signalling pathways responsible for the cytokine-induced PDGF and PDGF-R expression gives further possibilities to specifically interact with the effects exerted by PDGF and its receptors.

The cytokines in the present thesis have been added one-by-one to the cells. However, the *in vivo* situation is far more complex with a network of cytokines interacting with each other. Studies could therefore be extended to investigate combinations of different cytokines and their importance for the expression of PDGF and PDGF-R in various cell types.

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