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Regulation of arterial smooth muscle cell proliferation after endothelial injury

-an experimental approach to restenosis and transplant arteriosclerosis

by

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Academic Dissertation
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TABLE OF CONTENTS **Page**

ORIGINAL PUBLICATIONS 6

ABBREVIATIONS 7

INTRODUCTION 8

REVIEW OF THE LITERATURE 9

1. Vasculoproliferative disorders 9
1.1. Atherosclerosis 9
1.2. Restenosis 9
1.3. Transplant arteriosclerosis 10

2. Smooth muscle cells in the pathogenesis of atherosclerosis and restenosis 11
2.1. Experimental models for studying SMC migration and proliferation 11
2.2. Smooth muscle cell differentiation 12
2.3. Smooth muscle cell and macrophage apoptosis in arteriosclerosis and restenosis 13

3. Role of inflammation in vascular lesion formation 13
3.1. Endothelial adhesion molecules 13
3.2. Proinflammatroy cytokines 14
3.3. Peptide growth factors 14
3.4. IGF-1 15
3.5. PDGF 15
3.6. Regulation of local growth factor production 16

4. Intervention of growth factor action 17
4.1. General 17
4.2. Peptides and D-peptide analogs 17
4.3. Tyrosine kinase inhibitors 17

AIMS 19

METHODS 20

1. Experimental animals 20
2. Rat models of arteriosclerosis and restenosis 20
2.1. Ischemia-induced injury in the rat aorta 20
2.2. Rat carotid artery denudation 21
2.3. Rat aortic denudation and reinjury of the rat aorta 21
3. Semiquantitative RT-PCR 21
3.1. RNA isolation 21
3.2. RT-PCR 22
4. Histological evaluation 22
4.1. Determination of cell number 22
4.2. Morphometric analysis 23
4.3. Immunohistochemistry 23
4.4. Quantitation of immunohistochemistry 25
5. Cell kinetics studies in vivo 25
5.1. Quantitation of smooth muscle cell proliferation in vivo 25
5.2. Apoptosis assays 25
5.3. Quantitation of smooth muscle cell migration in vivo 26
6. Cell Culture studies 27
6.1. Primary smooth muscle cell Cultures 27
6.2. Rat coronary cell line 27
6.3. Quantitation of smooth muscle cell proliferation in vitro 28
6.4. Quantitation of smooth muscle cell migration in vitro 28
6.5. Tyrosine kinase assays 28
7. Drug administration and dosages 29
7.1. Recombinant human superoxide dismutase 29
7.2. D-peptide analogs 29
7.3. Tyrosine kinase inhibitors of the PDGF-R 29
8. Statistical methods 30

RESULTS 31

1. Lesion formation in rat arteries after injury 31
1.1. Cold-ischemia-induced arteriosclerosis 31

1.2. Rat carotid artery ballooning injury 32
1.3. Ballooning reinjury of the rat aorta 32

2. Inhibition of IGF-1 and PDGF receptors 33
2.1. Experimental models for studying smooth muscle cell migration and proliferation in the pathogenesis of arteriosclerosis and restenosis 33
2.2. PDGF-R tyrosine kinase inhibitor in rat carotid and aorta after single ballooning injury 34
2.3. PDGF-R tyrosine kinase inhibitor in ballooning reinjury of the rat aorta 34
2.4. Inhibition of IGF-1 and PDGF receptors in vitro 34

DISCUSSION 37

1. The response-to-injury hypothesis 37
2. Experimental models for studying vasculoproliferative disorders 37
3. Neointimal smooth muscle cells 38
4. Inflammation in arteriosclerosis and restenosis 39
5. Therapeutic strategies for vasculoproliferative disorders 40
6. Candidate drugs 41

SIGNIFICANCE OF FINDINGS 42

SUMMARY 43

YHTEENVETO (FINNISH SUMMARY) 46

SAMMANDRAG (SWEDISH SUMMARY) 48

ACKNOWLEDGEMENTS 50

REFERENCES 52

ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals:

I
Myllärniemi M, Räisänen-Sokolowski A, Vuoristo P, Kallio E, Land W and Häyry P. Lack of effect of recombinant human superoxide dismutase on cold-ischemia-induced arteriosclerosis in syngeneic rat aortic transplants. Transplantation 1996; 61: 1018-1022

II
Häyry P, Myllärniemi M, Aavik E, Alatalo S, Aho P, Yilmaz S, Räisänen-Sokolowski A, Cozzone G, Jameson B A, Baserga R. Stable D-peptide analog of insulin-like growth factor-1 inhibits smooth muscle cell proliferation after carotid ballooning injury in the rat. FASEB J 1995; 9: 1336-1344

III
Myllärniemi M, Calderón Ramirez L, Lemström K, Buchdunger E, Häyry P. Inhibition of platelet-derived growth factor receptor tyrosine kinase inhibits vascular smooth muscle cell migration and proliferation. FASEB J 1997; 11: 1119-1126

IV
Myllärniemi M, Rasilainen S, Lemström K, Häyry P. Enhanced intimal proliferation upon injury to pre-existing neointima and resistance of neointimal cells to apoptosis. Submitted

V
Myllärniemi M, Frösen J, Calderón Ramirez L, Buchdunger E, Lemström K, Häyry P. Selective tyrosine kinase inhibitor for the platelet-derived growth factor receptor in vitro inhibits smooth muscle cell proliferation after reinjury of arterial intima in vivo. Cardiovasc Drugs Ther 1999 (in press)

ABBREVIATIONS

bFGF, basic fibroblast growth factor
CD, cluster of differentiation
BrdU, Bromodeoxyuridine
DMEM, Dulbecco´s modified Eagle´s medium
DMSO, dimethyl sulphoxide
EGF, epidermal growth factor
FCS, fetal calf serum
HE, haematoxylin and eosin
3H-TdR, tritiated thymidine
ICAM-1, intercellular adhesion molecule 1
IGF-1, insulin-like growth factor 1
IGF-1R, IGF-1 receptor
LFA-1, lymphocyte function associated antigen 1
PDGF, platelet-derived growth factor
PDGF-R, PDGF receptor
PTCA, percutaneous transluminal coronary angioplasty
rhSOD, recombinant human superoxide dismutase
RT-PCR, reverse transcriptase polymerase chain reaction
SMC, smooth muscle cell
TGF-ß1, transforming growth factor ß1
TUNEL, terminal dideoxynucleotide transferase-mediated gig ddUTP nick end labeling
VCAM-1, vascular cell adhesion molecule 1
VLA-4, very late antigen-4

INTRODUCTION

Restenosis after percutaneous transluminal coronary angioplasty operations occurs in 30-40% of patients within 3-6 moths (Holmes et al., 1984). Chronic rejection remains the most important cause of late transplant loss in heart and kidney transplantations (Cecka and Terasaski, 1993). The biomechanisms of these vasculoproliferative processes still remain largely unknown, and there is no treatment for such diseases.

Neointimal formation leading to occlusion of arteries is the principal pathogenic mechanism underlying atherosclerosis, restenosis after angioplasty operations and transplant vascular sclerosis. The common feature in all these fibrointimal dysplasias is inappropriate neointimal smooth muscle cell (SMC) proliferation leading finally to occlusion of the artery. Several peptide growth factors have been identified in the formation of classical atherosclerotic (Ross, 1991), transplantation arteriosclerotic (Gordon, 1992) and restenotic (Cercek et al., 1991; Libby et al., 1992) plaques by inducing migration and proliferation of SMC. In addition to migration and proliferation, programmed cell death is involved in maintaining cell numbers in vascular wall layers (Björkerud and Björkerud, 1997). More recently, inflammation has been considered to be an important etiologic factor in neointimal lesion formation (Zhou et al., 1996). Inflammatory cells are abundantly found in atherosclerotic plaques and in the blood vessels of organ transplants, where they are believed to induce cytokine and adhesion molecule expression leading to the progression of disease.

This study focuses on the mechanism of neointimal formation in experimental rat models of endothelial injury caused either by prolonged cold ischemia time or a balloon catheter, and the regulatory roles of insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF) on the formation of these lesions.

REVIEW OF THE LITERATURE

1. Vasculoproliferative disorders

1.1. Atherosclerosis

Atherosclerosis is a chronic inflammatory, fibroproliferative disorder causing luminal narrowing of arteries and leading to oxygen insufficiency in peripheral organs. Our understanding of the pathogenesis of atherosclerosis has widely increased in the past decades (Ross et al., 1984, 1986, 1993, 1995). Hyperlipidemia, hypertension, glucose intolerance, and smoking are now commonly accepted as risk factors for atherosclerosis. So far, lipid-lowering treatment has been shown to be effective in the primary (Kjekshus and Pedersen, 1995; Sheperd et al., 1995) and secondary (Sacks et al., 1996) prevention of coronary events, such as myocardial infarction. Also, antiplatelet therapy has been shown clinically effective in the primary prevention of vascular occlusion (Antiplatelet Trialists´ Collaboration, 1994a), as well as in maintaining vascular patency after restenosis (Antiplatelet Trialists´ Collaboration, 1994b). More recently, certain inflammatory agents such as chlamydia pneumoniae (Kuo et al., 1993; Saikku et al., 1988) or cytomegalovirus (Chiu et al., 1997; Speir et al., 1994) have been identified as possible ethiologic factors for atherosclerosis and restenosis acting as local inducers of inflammatory activation in arteries. Interestingly, also macrolide antibiotic treatment effective on chlamydia pneumoniae infection, among others, has been shown in pilot studies to be effective in the secondary prevention of cardiovascular events (Gupta et al., 1997), while other prevention forms are being pursued.

1.2. Restenosis

Percutaneous transluminal coronary angioplasty (PTCA) is an effective noninvasive procedure for coronary revascularization relieving symptoms due to coronary arterioclerosis. However, restenosis after coronary angioplasty limits the long-term success of PTCA. The restenosis rate after angioplasty to stenosis or total occlusion of coronary arteries ranges from 30 to 40% (Holmes et al., 1984; Gruentzig et al., 1987), despite multiple preventive attempts with pharmacological or mechanical means. Restenosis is a hyperplastic, pathologic reaction involving SMC migration and proliferation, extracellular matrix formation and remodelling of the arterial wall leading

finally to reocclusion of the enlarged artery (Haudenschild, 1993). Similarly to early, fibrous atheromas, human restenotic lesions consist mainly of fibrocellular tissue (Virmani et al., 1994). Injury to endothelial and SMC caused by the angioplasty dilatation catheter followed by a proliferative response is believed to precede vascular remodelling and occlusion (Austin et al., 1985). So far, the mechanism and risk factors for restenosis remain largely unknown, and no effective treatment exists for restenosis. Some studies suggest (Tschopl et al., 1997), that high procoagulant factrors after angioplasty are risk factors for restenosis, and antiplatelet therapy is highly efficient in the reduction of vascular occlusion after angioplasty (Antiplatelet Trialists´Collaboration, 1994b). Also, promising preliminary results have been obtained with the antiproliferative agents such as competitive inhibitor of PDGF, triazolopyrimidine (Maresta et al., 1994) and angiopeptin (Emanuelsson et al., 1995). Angiopeptin is believed to be a suppressor of local growth factor production, and thus inhibit SMC proliferation (Häyry et al., 1993a).

1.3. Transplant arteriosclerosis

Chronic rejection or transplant arteriosclerosis remains the most important cause of late transplant loss in heart and kidney transplantations (Cecka and Terasaski, 1993). The lesions in transplantation arteriosclerosis resemble in their consistence the lesions of classical atherosclerosis (Billingham, 1992; Häyry et al., 1993b) although intimal thickening is concentric in comparison to the eccentric neointimal lesions in classical atherosclerosis, and calcification usually never occurs. Risk factors for chronic rejection are somewhat similar to the risk factors for atherosclerosis mentioned above. Apart from these, prolonged cold ischemia time and the generation of free oxygen radicals have been suggested to be risk factors for chronic rejection (Wanders et al., 1995). The generation of free radicals during reperfusion is a likely mechanism leading to endothelial damage and SMC replication in the arterial intima (Yilmaz, 1992; Flitter, 1993; Ward and Mc Carthy, 1994). Free oxygen radical scavengers, such as superoxide dismutase enzyme or antioxidant therapy have been considered as treatments for reperfusion injury (Land et al., 1994).

2. Smooth muscle cells in the pathogenesis of arteriosclerosis and restenosis

2.1. Experimental models for studying smooth muscle cell migration and proliferation in the pathogenesis of arteriosclerosis and restenosis

Rat carotid and aortic endothelial denudation are widely used models to study restenosis of arteries after balloon angioplasty. One of the weaknesses of these models is that the endothelial injury is induced on a normal vessel, whereas in man interventions are made on vessels with pre-existing lesions (Schwartz et al., 1995). Also, no anticoagulant therapy is usually given to rats subjected to ballooning injury, whereas anticoagulation is an absolute requirement in humans whenever vascular interventions are performed. Remodelling or recoil of vessels after angioplasty may be of great importance in the pathogenesis of human restenosis, and therefore models in larger animals have been developed (Carter et al., 1994; Geary et al., 1996). On the other hand, similar proliferative patterns are seen in rodents and in primates (Geary et al., 1996). Two recent experimental studies indicate that rodent models of repeated ballooning injury may be more helpful than single injury models in determining the mechanisms of restenosis after balloon angioplasty (Stadius et al., 1996; Koyama and Reidy, 1997). As in angioplasty operations, ballooning in reinjury models is performed on an artery with a thickened intimal layer (a diseased vessel), and the proliferative pattern of neointimal cells is similar to the one seen in large animal models: a self-limited proliferative burst instead of a sustained one seen in single injury models.

Cell cultures of arterial SMC allow studies on the mechanisms of the regulation of SMC proliferation and migration in a closed system. Aortic transplantation between histoincompatible rat strains is a useful and widely used model for studying acute and chronic rejection in blood vessels (Mennander et al., 1991). In transplanted vessels, endothelial injury occurs due to hypoxic/ischemic injury or acute rejection. After the acute rejection has subsided in these grafts, transplanted vessels have the same morphological features as injured vessels. Syngeneic transplants allow us to study the effect of cold ischemia/reperfusion injury separately from the immunological events in transplanted organs. Syngeneic rat aortic transplants subjected to prolonged cold ischemia time resemble morphologically balloon injured aortas and have neointimal lesions consisting mainly of SMC α-actin positive cells (Wanders et al., 1995). It is likely that the mechanisms of lesion formation in all these models partially overlap.

Therefore, therapeutic strategies that suppress SMC proliferation in any of these models can be used in the treatment of the diseases with a similar pathogenesis.

2.2. Smooth muscle cell differentiation

Matrix metalloproteinase activity in the vessel wall is a requirement for extracellular matrix degradation and SMC migration (Bendeck et al., 1994, 1996). Cytokines released by inflammatory cells can induce matrix metalloproteinase expression and enhance SMC migration (Galis et al., 1995). The plasminogen-plasmin system and the urokinase type plasminogen inhibitor are rate-limiting regulators of metalloproteinase activity and cell accumulation after endothelial injury (Lijnen et al., 1998), and the presence of plasminogen activators is a requirement for optimal response to PDGF-induced mitogenic signals (Herbert et al., 1997). Regardless of the primary cause of the endothelial injury (i.e. accumulation of oxidized fat, mechanical and/or immunological injury), the response to injury in the vascular wall leads to proliferation of SMC in the medial and adventitial layers, migration of neoitimal SMC precursor cells to the intima, and subsequent proliferation therein (Ross, 1993). Fibrous (early) atherosclerostic lesions and restenotic lesions consist mainly of SMC (Fuster, 1997; Strong et al., 1997). It has also been suggested that mononuclear or hematopoietic stem cells derived from the circulation differentiate into intimal SMC (Bondjers et al., 1991).

Classically, it has been thought that SMC exist in two different phenotypes, of which the "resting", "contractile" phenotype is located predominantly in the medial layer, whereas the "synthetic" phenotype is the predominant SMC type in the neointimal lesions both in experimental models and in humans (Thyberg et al., 1983). Lesion progression is observed predominantly in SMC-rich lesions in comparison with macrophage-rich lesions, and these regions have a high content of synthetic phenotype SMC (Strong et al., 1997). Recent studies indicate that neointimal cells are in fact derived from different subpopulations of certain types of medial/adventitial cells and not by differentiating from *any* medial SMC (Cascells, 1992; Lemire et al., 1994; Frid et al., 1997). The gene expression of neointimal SMC resembles the one of SMC in developing blood vessels (Majesky et al., 1992; Dupláa et al., 1997).

2.3. Smooth muscle cell and macrophage apoptosis in arteriosclerosis and restenosis

Apoptosis, or programmed cell death is involved in the development of tissues and maintaining stable cell numbers in the epithelia. Apoptotic SMC and macrophages are found in atherosclerotic lesions, where they are believed to contribute to the risk of plaque rupture in developed lesions (Björkerud and Björkerud, 1997) as well as maintaining stable cell numbers in neointimal lesions (Isner et al., 1996; Kockx and DeMeyer, 1996). Little is known about apoptosis in restenotic lesions, although some studies suggest that apoptosis is even more frequent in advanced restenotic lesions than in atherosclerotic lesions (Isner et al., 1996). It would therefore be plausible that alterations in apoptotic activity could also lead to increase, or decrease, in plaque size and luminal occlusion of arteries.

3. Role of inflammation in vascular lesion formation

3.1. Endothelial adhesion molecules

One of the first changes in atherosclerotic lesion formation is the adherence of mononuclear inflammatory cells to the endothelial layer and the subsequent infiltration of these cells into the subendothelial space (Ross, 1995). Increased levels of activated leukocytes are present in the peripheral blood after coronary angioplasty, and a higher level of leukocyte activation correlates with late clinical events (Mickelson et al., 1996). The injured/regenerating endothelium directs the adherence of monocytes by expressing adhesion molecules that bind to specific receptors in the mononuclear inflammatory cells (Tanaka et al, 1993, 1994). Adhesion molecules are overexpressed in the endothelium overlying atherosclerotic plaques (O´Brien et al., 1993a) or chronic rejection -related vascular lesions (Koskinen and Lemström, 1997). Local inflammatory factors induce the expression of adhesion molecules on endothelial cells (Couffinhal et al., 1994). It is believed that these membrane glycoproteins mediate leukocyte rolling to the vascular wall (Sakai et al., 1997). Firm adhesion of leukocytes to the vasculature is further mediated by vascular cell adhesion molecule 1, VCAM-1, and intercellular adhesion molecule 1, ICAM-1. Recent findings indicate that these adhesion molecules are also expressed during SMC differentiation (Couffinhal et al., 1994; Dupláa et al., 1997). The significance of adhesion molecule expression during SMC differentiation is not known.

3.2. Proinflammatory cytokines

As mentioned above, there is increasing evidence of a local immune response in the pathogenesis of atherosclerosis. However, fewer reports exist concerning the inflammatory nature of human restenotic lesions. Activated mononuclear inflammatory cells infiltrating the vascular wall are capable of producing inflammatory mediators, such as TNF−α and IL-1, which contribute to the local immune reaction and activation of the vascular wall (Hancock et al., 1994). These cytokines are also produced by proliferating SMC (Tanaka et al., 1996), and they have been shown to induce SMC proliferation *in vitro* (Thyberg et al., 1990; Casscells, 1991). Cytokines in turn induce the expression of chemotactic and pro-proliferative peptide growth factors and their receptors possibly initiate autocrine cycles leading to persistent SMC proliferation and lesion progression (Raines et al., 1989; Battegay et al., 1990). Also, adhesion molecule expression such as VCAM-1 and ICAM-1 expression are induced by these same cytokines (Wang et al., 1994; Libby and Galis, 1995). However, the regulatory networks of these complicated interactions are so far unknown.

3.3. Peptide growth factors

Several peptide growth factors contribute to the formation of atherosclerotic and restenotic lesions (reviewed by Ross, 1991 and Cercek et al., 1991, respectively). The same growth factors induce SMC proliferation *in vitro* (Thyberg et al., 1990; Casscells, 1991), and are expressed by SMC (Casscells, 1991) or released from injured SMC (Crowley et al., 1995). Elevated levels of mRNA transcripts of IGF-1 (Cercek et al., 1990), EGF (Miano et al., 1993), PDGF A and B chains (Miano et al., 1993), transforming growth factor-ß1, (TGF-ß1) (Majesky et al., 1991), and several others, have been detected in the vascular wall after endothelial injury, and furthermore, antibodies to PDGF (Ferns et al., 1991), basic fibroblast growth factor (Lindner and Reidy, 1991), and TGF-ß1 (Kanzaki et al, 1995), prevent neointimal formation after endothelial injury demonstrating that these growth factors are essential in this process. The experiments in this study focus on the role of IGF-1 and PDGF in SMC proliferation in vitro and neointimal formation in vivo in the rat arteries.

3.4. IGF-1

IGF-1 is known as a major regulator of embryonic growth (Baker and Liu, 1993). Previously known as somatomedin for its effects as the major mediator of growth hormone actions, IGF-1 is abundant in the circulation and its action is regulated by six binding proteins (reviewed by Jones and Clemmons, 1995). Most of the cellular events of IGF-1 are mediated by its receptor, IGF-1R. It has recently become evident that IGF-1 is also an important regulator of proliferation of mesenchymal cells, including fibrobasts and SMC. Upregulation of IGF-1 has been documented after ballooning injury (Cercek et al., 1990). The optimal mitogenic responses to epidermal growth factor (EGF) and PDGF require a functional IGF-1R (Pietrzkowski et al., 1992; Miura et al., 1994) and IGF-1 and PDGF are known to act synergistically in the induction of cellular proliferation (Clemmons, 1985), possibly through upregulation of IGF-1R expression (Delafontaine et al., 1991; Rubini et al., 1994). Antisense oligonucleotides that inhibit expression of IGF-1R inhibit thrombin, angiotensin II, and PDGF-induced mitogenesis in vascular SMC (Delafontaine et al., 1996), indicating that the IGF-1R activation is a rate limiting step in cell cycle progression (Baserga, 1993). More recently, it has been shown that IGF-1R activation mediates chemotactic signaling in SMC (Bornfeldt et al., 1994).

3.5. PDGF

PDGF were initially discovered as growth factors released from platelets at sites of injury.They are now recognized to be produced also by macrophages and SMC in atherosclerotic lesions, and they are known to be among the most potent SMC chemoattractants and mitogens. There are two homologous PDGF polypeptides, the PDGF-A chain and the PDGF-B chain, which are found as disulphide-linked dimers PDGF-AA, -AB and -BB (Ross et al., 1986; Heldin and Westermarck, 1990). Two types of PDGF receptor chains are known, the α-and ß-receptors (PDGF-Rα and PDGF-Rß). PDGF-Rα binds all PDGF-isoforms, whereas the ß-receptor binds PDGF-BB with high affinity and PDGF-AB with lower affinity, but does not bind PDGF-AA (Hart et al., 1988). The binding of PDGF ligand to its receptor leads to dimerization of receptor subunits followed by activation of the receptor protein tyrosine kinase and receptor autophosphorylation (Heldin et al., 1989).

The *in vivo* role of the PDGF-A chain and PDGFR-α are not yet known, but could be linked to proliferation, as evidenced by colocalization of PDGF-A production and cellular proliferation in human atherosclerotic plaques (Rekhter and Gordon, 1994) and rat neointima (Majesky et al., 1990). Furthermore, *in vitro* studies suggest that PDGF-A acts mainly as a mitogen for vascular cells (Koyama et al., 1994a), and may have regulatory functions in relation to PDGF-Rß mediated signalling (Koyama et al., 1994b). PDGF-BB is known as the most potent chemoattractant and mitogen to SMC (Grotendorst et al., 1982; Heldin and Westermarck, 1990; Thyberg et al., 1990). Local overexpression of the PDGF-B gene is accompanied by migration and proliferation of SMC and thickening of uninjured arteries (Nabel et al, 1993) and administration of PDGF protein leads to enhanced migration of cells in rat carotid artery (Jawien et al., 1992). Inhibition of PDGF-Rß expression with antisense oligonucleotides dramatically suppresses neointimal thickening in rat carotid artery (Sirois et al., 1997).

3.6. Regulation of local growth factor production

After endotelial injury, mononuclear inflammatory cells adhere to sites of injury. CD4+ T-cells express inflammatory cytokines such as interleukin-1 and tumour necrosis factor-α, which in turn trigger SMC and macrophages to produce growth factors creating an autokrine cycle of growth factor production and proliferation. Apart from these paracrine factors, hormones regulate local growth factor production in the vascular wall. Growth hormone is known as the major regulator of IGF-1 production in the liver. Hypophysectomy decreases intimal SMC proliferation via downregulation of IGF-1 (Khorsandi et al, 1992). Somatostatin analogs, such as octreotide and lanreotide are inhibitors of growth hormone/IGF-1 axis, and inhibit SMC proliferation in vitro (Grant et al., 1994) and growth factor production in aortic transplats in vivo (Häyry et al., 1993). Ocreotide downregulates IGF-1 levels in arteries after injury, but has no significant effect on blood growth hormone levels (Yumi et al., 1997), indicating that somatostatin or its analogs act as a local regulator of IGF-1 production. Recent findings indicate that the estrogen receptor ß is a major regulator of neointimal proliferation (Mäkelä et al., 1999). Estrogen has been shown to be inhibitory to growth factor-induced proliferation. However, it is so far not known whether estrogen receptor ß regulates growth factor production, or whether it regulates the cell cycle independently from growth factor action.

4. Intervention of growth factor action

4.1. General

The interference of gowth factor/receptor interaction may be a suitable therapeutic means for the prevention of proliferative response to vascular injury. Several approaches have been developed for the inhibition of growth factor/ligand interactions, such as the inhibition of gene expression with antisense oligonucleotides (Bennett and Schwartz, 1995) and transfection of cells using liposomal or viral vectors (Nabel et al., 1993, 1997; Yonemitsu et al., 1997;) or inactivation of growth factors with blocking antibodies (Ferns et al., 1991; Lindner and Reidy, 1991; Wolf et al., 1994). However, these approaches are relatively experimental and have many disadvantages when therapeutic applications for humans are being developed.

4.2. Peptides and D-peptide analogs

One approach in rational drug design is to design and synthesize peptidic compounds that mimic natural protein conformations. However, normal L-amino acids are rapidly degraded by body proteases. D-amino acids are resistant to this proteolytic degradation in body fluids and thus have longer half-lives (Brady and Dodson, 1994). The conformation of D-amino acids being a mirror image to normal body proteins render them incapable of conformation-dependent interactions with body proteins. If, however, synthesised in reverse order, D-peptidic analogs mimic the conformation of natural L-peptides. With this approach, activation of CD4+ T cells (Jameson et al., 1994) or IGF-1/IGF-1R (Pietrzkowski et al., 1992) interactions can be competitively inhibited. These D-peptidic analogs may become important tools in studies of the pathogenesis of disease processes and finding possible therapeutic applications.

4.3. Tyrosine kinase inhibitors

Promising tools for the inhibition of growth factor mediated signal transduction in the cell are receptor tyrosine kinase inhibitors. Tyrosine kinases that are overactivated by a disease process can be specifically inhibited, and systemic side effects thus can be avoided. Individual receptor tyrosine kinase conformations in different receptor families opens the possibility for the development of selective tyrosine kinase inhibitors (Levitzki and Gazit, 1995; Levitzki, 1996). Nonselective tyrosine kinases have the

disadvantage of inhibiting normal receptor tyrosine kinase-mediated growth thus rendering them toxic when systemically administered. This problem can partially be overcome by local administration (Kozai et al, 1997). However, the existence of highly selective tyrosine kinase inhibitors i.e., for EGF receptor, PDGF-R (reviewed by Kovalenko et al., 1997), and IGF-1R (Parrizas et al., 1997) may allow systemic administration.

AIMS

The hypothesis in this study was, that peptide growth factors are major regulators of vascular SMC migration and proliferation. Since peptide growth factors are abundant in tissue and circulation, their receptors are more likely regulators of local cellular events, such as migration and proliferation of vascular SMC, and thus more specific targets for therapy. The aims of this study were:

1. To study SMC proliferation, migration and apoptosis in the formation of neointimal lesions in rat models of arteriosclerosis and restenosis.

2. To investgate the role of PDGF or IGF-1-induced receptor activaction in vascular SMC migration and proliferation.

In order to study arteriosclerotic lesion formation, three experimental rat models were used: syngeneic rat aortic transplantations, ballooning injury of the rat carotid artery, and reinjury of the rat aorta. For inhibition of lesion growth induced by IGF-1, a D-peptide analog of IGF-1, JB3 was used. For inhibition of PDGF-R activation, a tyrosine kinase inhibitor, CGP 53716, and its water soluble derivative, CGP 57148B were used.

METHODS

1. Experimental animals

Male Wistar/SD rats or inbred WF (AG-B2 RT^{1u}) or DA (AG-B4, RT^{1a}) rats purchased from the Laboratory Animal Centre, University of Helsinki, Helsinki, Finland were used for the studies. A permit for animal studies was approved by the Municipal Government of Uusimaa (KUN 78). Laboratory rats were treated according to the Finnish law on animal rights (9§ 777/85). All animals received humane care in compliance with the principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared and formulated by the National Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication no. 86-23) and the European agreement for the use of experimental animals in scientific research. The rats were anesthesised with chloralhydrate, 240 mg/kg ip., and 0.3 mg/kg sc. buprenorphine (Temgesic, Reckitt & Colman, Hull, England) was used for postoperative pain relief. A permit for the use of chloral hydrate anesthesia was received from the county veterinarian. The basic diet of the animals was pellets (Altromin N:o. 1314, Standard diet, Chr. Petersen A/S, Ringsted, Denmark) and they were given tap water.

2. Rat models of arteriosclerosis and restenosis

2.1. Ischemia-induced injury in the rat aorta

For studies on cold-ischemia induced arteriosclerosis, the rat aortic transplantation model of inbred DA rat strain was used. The surgical procedure was performed as described by Mennander et al. (1991). The descending thoracic aorta was excised, perfused with phosphate buffered saline and used as a transplant. The graft was sutured into a heterotopic position below the renal arteries in the abdominal aorta. Syngeneic grafts with prolonged cold ischemia were stored for 24 h in 4°C phosphate buffered saline before transplantation. The grafts were removed at 1, 2, 3 or 6 mo after transplantation. Some grafts were analysed immediately after cold preservation. Syngeneic transplantations with short ischemia times (5-15 min) served as controls.

2.2. Rat carotid artery denudation

The left common carotid artery was denuded of endothelium by the intraluminal passage of a 2 French Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Santa Ana, CA) introduced through the external carotid artery as described (Clowes et al., 1983). The catheter was passed three times with the balloon inflated with 0.2 ml air. The external carotid artery was ligated and the wound was closed.

2.3. Rat aortic denudation and reinjury of the rat aorta

The thoracic aorta was denuded of endothelium using a 2 French Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation). The catheter was introduced into the thoracic aorta via the left iliac artery, inflated with 0.2 ml air, and passed five times to remove the endothelium. The iliac artery was ligated after this procedure. When a second injury was produced, the catheter was introduced to the aorta upstream from the initial ligature, and the procedure was repeated in a similar manner as for the first injury. The animals were harvested at 0, 3, 7 and 14 d after both injuries and their tissues were processed for histology and immunohistochemistry. No anticoagulant therapy was given to the rats.

3. Semiquantitative RT-PCR

3.1. RNA isolation

Biopsies taken from blood vessels were snap-frozen immediately in liquid nitrogen. Total cellular RNA was extracted by the guanidium isothiocyanate method (Davis et al, 1986). Tissues were homogenized (Ultra-turrax, Janke & Kunkel, Staufen, Germany) in 3 ml guanidium-isothiocyanate buffer. The homogenate was pipetted on 2 ml of 5.7 M CsCl buffer in polyallomer tubes (Beckman Instruments, Fullerton, CA). The tubes were centrifuged at 32 000 RPM for 21 h. After centrifugation, the samples were resuspended in TES buffer (10 mM Tris, pH 7.4, 5 mM EDTA, pH 7.4, 1% SDS) and phenol purified three times. Aqueous RNA was precipitated with 3 molar sodium acetate and the optical density was measured spectrophotomerically.

3.2. RT-PCR

First-strand cDNA was generated from 1 µg RNA in a 20 µL volume using commercial Perkin Elmer Cetus protocol (Norwalk, Conn.). One µl of the reverse transcription reaction mix was amplified with primers specific for rat IGF-1, PDGF-B, EGF, TGF-ß1, acidic and basic fibroblast growth factors in a total volume of 50 µl. The primer and probe sequences used are shown in paper II. The PCR-product was added to PCR-buffer (500 mM, Tris, pH 8.8, 15 mM $MgCl_2$, 150 mM NH4SO4, 0.1% gelatin, 1 % TRITON-X-100) using 0.2 mM of each nucleotide and 1 µM of each primer and 2.5 U Taq DNA-polymerase. The samples were processed for 25-28 cycles each with the following thermal parameters: 94 ^{o}C, 1 min, 57°C, 1 min and 72°C, 2 min. Glyseraldehyde-phosphate dehydrogenase (GAPDH) primers were used as the control PCR. The samples were electrophoresed on an agarose gel and Southern blotted to a nylon membrane. Radiolabeled probes specific for each growth factor were hybridized to the membrane and autoradiography was performed. The intensity of hybridization signals was measured with densitometry (Electrophoresis Data Center, Helena Laboratories, Beamont, TX).

4. Histological evaluation

4.1. Determination of cell number

Normal, noninjured rat arteries do not have a cellular neointimal layer, as do humans. The intima in intact rat arteries consist only of the internal elastic lamina extracellular matrix covered by the endothelial cell layer. For evaluation of morphological changes, aortic and carotid cross sections were fixed in 3% paraformaldehyde (pH 7.4), embedded in paraffin for sectioning, and stained with Mayer´s haematoxylin and eosin (HE). For evaluation of intimal and medial thickness and cell number in syngeneic aortic transplants, a microscope ocular with a 0.02 mm grid was used. The data was expressed as point score units, i.e., the mean number of points falling over a given anatomical area using 8 straight cross-sectional lines. The absolute number of cells in the intima, media and adventitia were calculated from paraffin cross sections at 400 x magnification.

4.2. Morphometric analysis

To quantitate neointimal/medial areas at the end of observations in the aortic denudation specimens, the intimal and medial areas were quantitated using Macintosh NIH image software. The picture from the microscope was transferred to a screen with an Olympus video microscope using 100x magnification. The areas inside the internal elastic lamina, the external elastic lamina, and of the aortic lumen were measured, and intimal and medial areas, and intima/media area ratio were calculated from these values.

4.3. Immunohistochemistry

Serial frozen sections (4-6 µm) were air-dried on silane coated slides, fixed in acetone at -20°C for 20 min, and stored at -20°C until used. Before immunostaining, the slides were refixed with chloroform and then air-dried. After incubation with appropriate 1.5% nonimmune serum (Vector Laboratories, Burlingame, CA) for monoclonal or polyclonal antibodies, frozen sections were incubated with a mouse monoclonal antibody at room temperature for 30 to 60 min or with a rabbit polyclonal antibody at +4°C for 12 h. The primary antibodies were diluted in PBS with 1% BSA and appropriate 3% nonimmune serum. With intervening washes in Tris-buffered saline, the following steps were performed: biotinylated horse anti-mouse or goat anti-rabbit rat-absorbed antibodies at room temperature for 30 min; avidin-biotinylated horse-radish complex (Vectastain Elite ABC Kit, Vector Laboratories) in PBS at room temperature for 30 min; the reaction was revealed by chromogen 3-amino-9-ethylcarbazole (AEC; Sigma) containing 0.1% hydrogen peroxidase, yielding a brown-red reaction product. The coverslips were counterstained with haematoxylin and mounted. The antibodies used are presented in Table 1.

Table 1. Mono- and polyclonal antibodies used to detect cell type, activation state, adhesion molecules and PDGF ligands/receptors.

Antibody against		Dilution	Manufacturer
mouse monoclonal	ED3 (activated MF)	1:200	Serotec, Oxford, UK
	CD4+ T-cells	1:100	W3/25, Sera-lab, Sussex, UK
	CD8+ T-cells	1:100	OX8, Sera-Lab
	Natural killer cells	1:100	3.2.3.*
	Class II	1:100	OX6; Sera-lab
	ICAM-1	1:100	CD 54, Seikagu, Tokyo, Japan
	LFA-1, lymphocyte function associated antigen 1	1:100	Seikagu
	VLA-4 very late antigen 4	1:100	Seikagu
	VCAM-1	1:100	Biogen, Cambridge, MA **
	P-selectin (CD62)	1:100	Pharmingen, Hamburg, Germany
	α-actin	1:500	Bio-Makor, Revohot, Israel
rabbit polyclonal	PDGF-AA	1:100	ZP214, Genzyme, Cambridge, MA
	PDGF-BB	1:100	ZP215, Genzyme
	PDGF-Ra	1:100	sc-338, Santa Cruz Biotecnology,
	PDGF-Rβ	1:100	sc-339; Santa Cruz Biotechnology,

* A generous gift from Dr. William H Chambers, Pittsburgh Cancer insititute, Pittsburg, P.A
** A generous gift from Dr Roy Lobb, Biogen, Cambridge, MA

Controls were performed using the same immunoglobulin concentrations of species and isotype-matched antibodies: mouse monoclonal IgG1 antibody (catalog No. X931; Dako, Glostrup, Denmark) and rabbit polyclonal immunoglobulin fraction (catalog No. X936; Dako) for monoclonal and polyclonal antibodies, respectively. Additional specifity controls for PDGF ligand and receptor staining involved the use of a working dilution of the polyclonal antibody after overnight incubation with a 10 to 20-molar excess of recombinant peptide (for Santa Cruz's antibodies). None of these control staining showed any immunoreactivity.

4.4. Quantitation of immunohistochemistry

For inflammatory cell and adhesion molecule staining, positive cells in each cross section were counted separately from the advential, medial, and intimal layers. The intensity of immunohistochemical staining for PDGF receptors and ligands was scored from 0 to 3 as follows: 0, no visible staining, 1, faint staining, 2, mild to moderate staining, and 3, intense staining.

5. Cell kinetics studies in vivo

5.1. Quantitation of smooth muscle cell proliferation in vivo

Tritiated thymidine (3H-TdR, Amersham International plc, Amersham, UK) was administered by iv. injection via the femoral vein to rats, at a dose of 50 µCi/100 g 3 h prior to sacrifice. Histology was processed from paraffin cross sections and emulsion film autoradiography (Ilford L-4; Ilford, Mobberley, Cheshire, England) was performed. Alternatively, thymidine analog Bromodeoxyuridine (BrdU, Zymed Laboratories, San Francisco, CA) labeling was used for quantitation of cellular proliferation. The rats received 0.3 ml iv. BrdU labeling reagent according to the manufacturer´s instructions three to six hours before sacrifice. Proliferating cells, which had incorporated BrdU were visualized by immunoperoxidase staining made from paraffin cross sections. The number of labeled nuclei was counted separately in the intima, media and adventitia of the sections.

5.2. Apoptosis assays

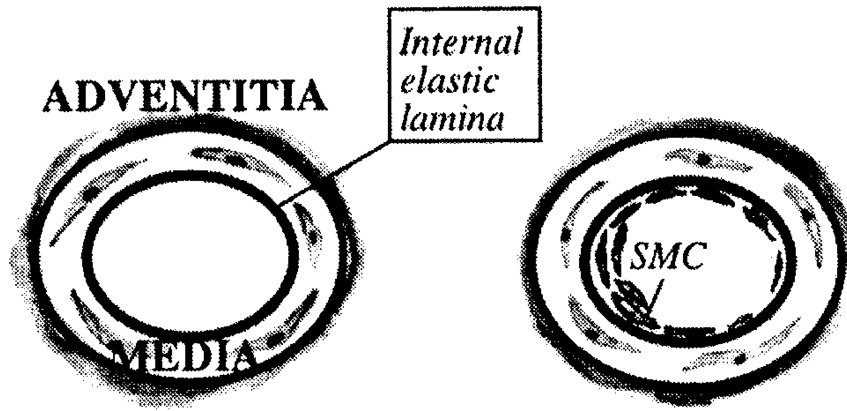
Programmed cell death was quantitated from rat aortic paraffin sections that had undergone one or two ballooning injuries. The sections were deparaffinized in xylene and alcohol and stained using the terminal dideoxynucleotidetransferase (TdT)-mediated digoxigenin-ddUTP nick end labelling (TUNEL) procedure. Reagents were purchased from Boehringer Mannheim (Mannheim, Germany). The deparaffinized sections were permeabilized by microwave treatment (for 5+3 min, in 10 mM citric acid), followed by preincubation in 5 mM $CoCl_2$ TdT buffer for 10 min (37°C) and the digoxigenin-ddUTP labelling of the nicked DNA ends by TdT for 60 min (37°C, 5 mM $CoCl_2$, 5 mM TdT-buffer, 0.23 mM ddATP, 0.13 mM dig-ddUTP, 0.58U/µl TdT). To detect the labeled cells, the sections were blocked by 2% blocking reagent in 150 mM NaCl, 100

mM Tris-HCl, and then treated with anti-digoxigenin Fab fragments, 0.19 U/ml in blocking buffer, 37˚C, 60 min. Finally, the sections were incubated with a peroxidase dye, NBT/BCIP solution in 67% DMSO, for up to 60 min. Nuclear counterstaining with hematoxylin was performed. A similar procedure without the Tdt-treatment was used as a negative control for every sample. Positive cell numbers were calculated separately from the intimal and medial layers from nondenuded control specimens and at 4 h, 3 and 7 days after the first and 4 h and 3 days after the second injury. The percentage of apoptotic cells was calculated from the total number of TUNEL+ cells compared to the total number of cells/cross section.

5.3. Quantitation of SMC migration *in vivo*

Based on the finding that the first SMC appear in the neointima 4 days after denudation of rat carotid artery, the *in vivo* migration rate of SMC was estimated using histological cross sections of denuded carotid specimens (Figure 1). BrdU labeling at different time periods after endothelial injury was used to exclude overestimation of the migration rate due to cell proliferation. For quantitation of SMC proliferation before any migrated cells are seen in the intima, 0.1 ml of BrdU-labeling reagent (Zymed) was administered i.v. every 12 h during postoperative hours 0-72 (a total of six x 0.1 ml). For quantitation of proliferation during the initial wave of migration, the rats received 0.1 ml BrdU labeling reagent three times at 8 h intervals during postoperative hours 72-96. For quantitation of proliferation at the end of the initial wave of migration, a third group of rats received a 0.3 ml BrdU pulse three hours before sacrifice. Using BrdU labeling during the initial wave of migration of SMC from the media into the neointima (72-96 h after injury), a reasonable estimate of the migration rate can be made (Calderón et al., unpublished observations).

Figure 1. In vivo migration assay. 72 h after carotid denudation, no cells are seen on the luminal side of the internal elastic lamina. 96 h after injury, the first cells appear in the neointima. This "window" was used to quantitate migration of α-actin positive cells.



6. Cell culture studies

6.1. Primary smooth muscle cell cultures

Primary aortic SMC were isolated from 9 to 11-day-old DA (AG-B4, RT1^{a}) rat aortas using a method modified from that of Thyberg et al. (Thyberg et al., 1983). The aortas were opened longitudinally and the endothelial layer was gently scraped off. The adventitia and media were separated, and the medial layer was digested with 0.1% collagenase and DNAse in PBS for 30 min at +37°C. The cells were centrifuged, suspended in culture medium and allowed to attach to plastic flasks. Primary cells were used at passage 2-6 for the experiments. The cells were subcultured in Dulbecco´s modified eagle´s medium (DMEM, Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS), 2 M glutamine, 100 M streptomycin and 100 IU/ml penicillin. For identification, the cells were grown on glass coverslips and stained for SMC α-actin (as described above).

6.2. Rat coronary cell line

Alternatively, a rat coronary SMC line from Dr. C. A. Diglio (Wayne State University, Detroit, MI) kindly provided by Dr Dariusz Leszczynski from the State Department of Radiation Hazard, Helsinki, were used for the in vitro experiments. The cells were subcultured in DMEM supplemented with 10% FCS, 2 mmol/ml glutamine, 100

mmol/ml streptomycin and 100 IU/ml penicillin. For identification, the cells were grown on glass coverslips and stained for SMC α-actin.

6.3. Quantitation of smooth muscle cell proliferation in vitro

Subcultures of SMC isolated from rat coronary artery, or primary cultures of baby rat aortic SMC were used. Cells were cultured in Dulbecco´s modified eagles medium supplemented with 10% FCS. For the experiments, cells were trypsinized and 8000 cells were seeded on 96-well multidishes (Nunc, Roskilde, Denmark) or culture slide microwells (Falcon, Beckton Dickinson, New Jersey) at a concentration of 40 000 cells/ml, and allowed to adhere to the wells for 24 h. After 72 h of serum starvation, quiescent cells were stimulated with growth factors (PDGF, IGF-1, EGF or bFGF) in serum-free medium for 24 or 42 h. The number of cells was quantitated by counting the cells microscopically (from culture slides) or by measuring Tritiated thymidine (3H-TdR; Amersham International plc, Amersham, UK) incorporation. Alternatively, SMC were seeded in 96-well multidishes (8000 cells/well) in full culture medium supplemented with 10% FCS. The cells were exposed daily to 1 µCi/ml of 3H-TdR or tritium-labeled glycine (3H-glycine; NEN Chemicals, Boston, MA) for 24 h to quantitate DNA and protein synthesis, respectively.

6.4. Quantitation of SMC migration in vitro

Migration was quantitated using Transwell culture chambers. SMC (one million/ ml) were seeded in the upper chamber, and the chemoattractant was added to the lower chamber. After an appropriate time (4-24 hours) the cells that had migrated to the lower side of the filter were fixed in methanol (+4°C), stained, and counted microscopically.

6.5. Tyrosine kinase assays

For measurement of PDGF-R tyrosine phosphorylation, Swiss 3T3 cells provided by Dr. G. Thomas (Friedrich-Miesher Institute, Basel, Switzerland) were used. Confluent cells were incubated for 90 min with CGP 53716 and PDGF-AA or PDGF-BB for 10 min. Cell lysate proteins were analysed by Western blotting using anti-phosphotyrosine antibodies. For measurement of IGF-1R phosphorylation, primary rat SMC were serum starved overnight, stimulated with 20 ng/ml IGF-1 and immunoprecipitated with anti-

phosphotyrosine antibodies. Bound antibodies were detected using the ECL Western blotting system from Amersham (Buckinghamshire, UK).

7. Drug administration and dosages

7.1. Recombinant human superoxide dismutase

Free radical scavenger recombinant human superoxide dismutase (rh-SOD) was administered to rats transplanted with a syngeneic graft subjected to cold ischemia in order to prevent reperfusion injury. administration of Rh-SOD was performed exactly as in a previous study with human kidney allografts, where 200 mg (approximately 3 mg/kg) of rh-SOD was given intravenously during 10 minutes of graft reperfusion (Land et al., 1994). Rh-SOD (Liposome Company, Princeton, NJ) was dissolved in PBS and given 3 mg/kg i.v. in a lumbar vein. The infusion was started one minute before reperfusing the graft and continued for ten minutes. Control rats were given a PBS-infusion.

7.2. D-peptide analogs

The D-amino acid peptide JB3 (C-S-K-A-P-K-L-P-A-A-Y-C) was synthesized using standard solid-phase techniques at the Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia as previously described by Pietrzkowski et al. and sufficient quantities for the experiment were provided by Raggio-Italgene, Rome, Italy. Before rat carotid denudation, 0.3 μg of JB3 was administrated s.c., and the remaining JB3 was given via Alzet mini-pumps (Alzet Corp., Palo Alto, CA) at the indicated dose rate for a period of 14 days. Four dosage rates of 10, 30, 100 and 300 μg/kg/day were given to individual rat groups, with at least five rats in each group. The impact of drug administration was compared to similarly operated rats receiving only diluent (saline). For in vitro assays, JB3 was added to 10% FCS medium or to serum-free medium supplemented with PDGF-BB at different concentrations (0.01-10 μg/ml).

7.3. Tyrosine kinase inhibitors of PDGF-R

Selective tyrosine kinase inhibitors CGP 53716 and CGP 57148B were synthezised by Novartis pharmaceuticals, Basel, Switzerland). Both compounds were administered in vivo at the same dose of 50 mg/kg/day. For *in vivo* studies, water-insoluble CGP 53716

was dissolved in dimethyl suphoxide at a concentration of 200 mg/ml and diluted thereafter 1:20 with 1% Tween in 0.9% NaCl. After sonication a homogenous solution was obtained. Stock solutions and dilutions were prepared daily prior to administration. This stock solution was diluted further in cell culture medium and used for the experiments at concentrations of 10-0.1 μM. A water soluble derivative of CGP 53716; CGP 57148B was dissolved in distilled water and administered orally once daily at a dose of 50 mg/kg/day. For *in vitro* studies, a 10 mM stock solution in DMSO (CGP 53716) or distilled water (CGP 57148B) was prepared. These stock solutions were diluted in cell culture medium, and used for the experiments at concentrations of 10-0.1 μM.

8. Statistical methods

In general, nonparametric tests were used for evaluation of statistical significance. Nonparametric tests were chosen because the requirement of normal distribution in nonparametric tests was usually not fulfilled due to a relatively small sample size. For evaluation of statistical significance between two treatment groups, Mann-Whitney U test was used. For evaluation of statistical significance between several groups, a combination of the nonparametric Kruskall Wallis test and Dunn test, or analysis of variance were used.

RESULTS

1. Lesion formation in rat arteries after injury

1.1. Cold-ischemia-induced arteriosclerosis (I)

Syngeneic DA-DA aortic transplants with prolonged cold ischemia time were stored for 24 h in 4° PBS before transplantation. Control grafts were transplanted with minimal ischemia times (5-15 min). The grafts were removed and analysed 0 h, 1, 2, 3 or 6 mo after transplantation.

Immediately after cold preservation, the grafts resembled normal aortas with the exception of an apparent reduction in endothelial cell numbers. Medial smooth muscle cells were present at that time. However, already after 1 mo post transplantation, severe loss in medial cell number (medial necrosis) was observed, which progressed for 3 mo and was not restored after 6 mo of observation. Slight medial cell loss was observed in the control group with a short ischemia time. Severe increase in neointimal cell numbers was observed already at 1 mo after transplantation in the prolonged cold ischemia group, whereas no significant increase in intimal cell number was observed in the control group.

In order to study the possible growth factors mediating neointimal formation and remodelling after prolonged cold ischemia time, semiquantitative PCR for known inducers of SMC proliferation was performed on RNA isolated from these grafts. At this time point, elevated levels of IGF-1 mRNA were detected, whereas levels of other growth factors remained unchanged (PDGF-B, TGF--ß1, aFGF and bFGF) or decreased (EGF).

One series of transplants were treated intravenously with rh-SOD in order to study whether treatment with this free radical scavenger would prevent ischemia-induced reperfusion injury and arteriosclerosis in aortic transplants. However, no effect on medial necrosis or intimal thickening was observed with the treatment regimen used.

1.2. Rat carotid artery ballooning injury (II-III)

After rat carotid artery ballooning injury, an initial increase in the medial cell number on day 2-3 was observed, which was followed by a gradual increase in intimal cell number peaking on day 14 after injury. The first neoinimal cells appeared in the neointima from 72 h after injury onwards. The number of SMC appearing in the neointima during 0-96 h was used as an estimate of the first wave of migrating cells after endothelial injury in the rat.

Medial cell proliferation was measured by BrdU and 3H-TdR incorporation, on days 3-5 after injury. When labeled continously during the first 3 days, 16 % of all medial cells had incorporated BrdU. Intimal cell proliferation peaked at day 7 after injury followed by a decrease in the number of proliferating cells.

In order to study the possible growth factors mediating SMC proliferation and neointimal formation after carotid ballooning injury, semiquantitative RT-PCR for known inducers of SMC proliferation was performed on RNA isolated from carotid arteries after injury. Elevated levels of IGF-1, PDGF-B and EGF were seen throughout the observation time from day 5 to day 100, peaking 5-7 days after injury. A slight peak increase in the level of TGF-ß1, acidic and and basic fibroblast growth factors were found approximately on day 7 after injury.

1.3. Ballooning reinjury of the rat aorta (IV-V)

In order to study the effect of ballooning injury to the neointimal instead of the medial SMC, a second ballooning injury was performed to rat thoracic aorta 14 d after the first injury. The absolute number of cells, proliferating (BrdU positive) cells, inflammatory cells and expression of adhesion molecules and PDGF receptors and ligands in different vascular compartements were studied from aortas 0, 3, 7 and 14 d after the first and second ballooning injury. Three days after the first injury, medial cell number increased, whereas no change in medial cell number was seen after the second injury. After the first injury, intimal cell number increased gradually. After the second injury a more prominent and accelerated increase in intimal cells was seen. A peak in cellular proliferation was seen in the medial layer at day 3 and in the intima at day 7 after the first injury, whereas after the second injury, a pronounced proliferative peak was seen already at day 3 after injury.

TUNEL staining for apoptotic cells of the critical time points of peak proliferation after the first and second injuries (0, 7 and 3 days) was performed in order to study the level of programmed cell death as a possible cause for the differential proliferative response observed after first vs. second injuries. At days 3 and 7, the ratio of apoptotic cells was 5 and 6%, respectively (P=NS.) The number and percentage of apoptotic cells was significantly higher 4 h after the first injury (23%) when compared to any other, but at 4 h after the second injury in the intima, it was only 2.6%, $P<0.01$.

Adhesion molecule VCAM-1 was upregulated in the medial and neointimal SMC during the proliferative peaks after both the first and second injuries. P-selectin was expressed in the regenerating endothelium after both injuries, whereas no P-selectin expression was observed in noninjured arteries. An increase in the CD4+ T cells, NK cells and activated mononuclear cells was observed in the adventitial layer after the first injury. After the second injury, a prominent increase in the number of CD4+ T cells and ED3+ activated macrophages was seen in the intima.

After the first injury, a sustained elevation of PDGF-AA, PDGF-BB, and PDGF-Rß was detected by immunohistochemistry in the media. Three days after the second injury, higher levels of expression of PDGF-AA and PDGF-R were observed.

2. Inhibition of IGF-1 and PDGF receptors

2.1. Effect of administration of D-peptide analog of IGF-1 in rat carotid artery (II)

D-peptide analog of IGF-1, JB3, was used to determine the impact of inhibition of IGF-1/IGF-1R interaction to lesion formation in rat carotid arteries. JB3 was administered as a continuous subcutaneous infusion to rats over 14 d after carotid ballooning injury at dosages of 10, 30 and 100 µg/kg/day. At dosages of 10 and 30 µg/kg/day, the compound significantly reduced the number of proliferating neointimal cells as measured by 3H-TdR incorporation. However, no significant effect was observed in intimal thickness 14 d after injury.

2.2. PDGF-R tyrosine kinase inhibitor in rat carotid and aorta after single ballooning injury (III)

Two PDGF-R tyrosine kinase inhibitors were used in order to study the effect of PDG-R tyrosine kinase activation after ballooning injury, CGP 53716 and its water-soluble derivative, CGP 57148B. Both of these compounds were shown to be specific inhibitors of the PDGF-R tyrosine kinase.

CGP 53716 was administered to rats ip. once daily at a dose of 50 mg/kg/day. After carotid denudation, CGP 53716 had no significant effect on the percentage of BrdU-incorporating adventitial, medial or intimal cells, neither when the cells were labeled during 0-72 or 72-96 postoperative hours. However, pulse labeling of the rats during the final 93-96 hours before sacrifice showed a marginal inhibitory effect on the percentage of BrdU-incorporating cells in the adventitia and media ($p<0.05$, Mann Whitney U test) though not in the intima. On the other hand, migration of α-actin expressing SMC from the media into the neointima during the time bracket of 72-96 hours was strongly inhibited from 38±10 (n=18) to 4±2 (n=18) nuclei per vessel circumference ($p<0.0001$), and there was a slight reduction in the number of cells in the adventitia and media ($p<0.05$). After rat aorta denudation, a significant decrease in the number of intimal cells and in the intima/media ratio was observed at 14 d ($p=0.03$).

2.3. PDGF-R tyrosine kinase inhibitor in ballooning reinjury of the rat aorta (V)

Finally, we investigated whether the enhanced proliferative response observed after repeated ballooning injury to the rat aorta could be interfered with by inhibition of PDGF-R tyrosine kinase. Peroral administration of the tyrosine kinase inhibitor CGP 57148B *in vivo* resulted in a 60% reduction both in the absolute number of BrdU+ cells and the percentage of BrdU+ cells 3 d after the second ballooning injury, but had no significant effect on proliferation after the first injury.

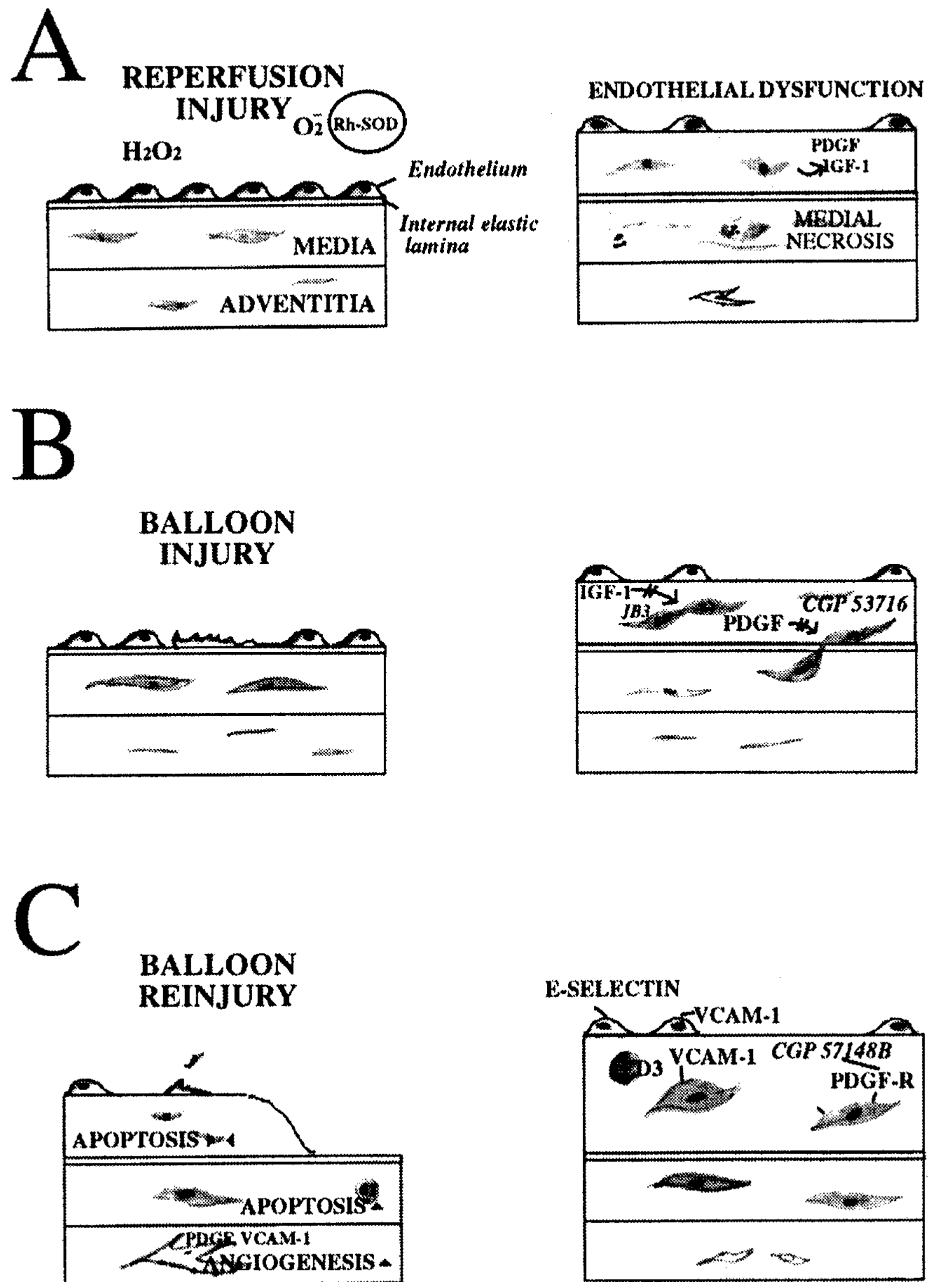
2.4. Inhibition of IGF-1 and PDGF receptors in vitro (II, III, V)

The D-peptide analog of IGF-1, JB3 inhibited the IGF-1-induced tyrosine phosphorylation in primary rat aortic SMC at a concentration range of 0.1-10 µg/ml. Maximal inhibition was seen at 1 µM concentration. Using Swiss 3T3 cells, shown to

express PDGF-receptors, tyrosine kinase inhibitor CGP 57316 inhibited PDGF-induced tyrosine phosphorylation of both receptor subtypes at an IC 50 value of approximately 0.03-0.1 μM.

In vitro, JB3 inhibited serum-induced primary rat aortic SMC 3H-TdR-incorporation in a biphasic manner, maximally at a concentration of 1 μg/ml. CGP 53716 inhibited PDGF-AA and PDGF-BB -induced 3H-TdR -incorporation and absolute cell numbers at 1 μM concentration dose-dependently. CGP 57148B selectively inhibited PDGF-AA and PDGF-BB -induced proliferation at the optimal 1 μM concentration, whereas IGF-1 and bFGF -induced proliferation were unaffected.

Figure 2. Summary of the major findings. (A) Recombinant human superoxide dismutase -infusion during aortic graft reperfusion was inefficient in inhibiting neointimal lesion formation in syngeneic aortic transplants. (B) After rat carotid ballooning, inhibition of IGF-1R with the D-peptide analog JB3 led to a slight decrease in lesion size possibly acting via inhibition in cellular proliferation, whereas inhibition of the PDGF-R with the tyrosine kinase inhibitor CGP 53716 inhibited mainly cellular migration. (C) After reinjury to rat aorta, a minimal apoptotic response was seen in the neointima, followed by an enhanced proliferative and inflammatory response. PDGF-R tyrosine kinase inhibitor CGP 5714BB inhibited this enhanced neointimal proliferation and end-point lesion formation.



DISCUSSION

1. The response-to-injury hypothesis

Neointimal formation and luminal occlusion of arteries is believed to be a pathological response to endothelial injury (reviewed by Ross, 1993). In addition to pure mechanical injury, caused by an angioplasty catheter, various other forms of injury can be identified as initiators of neointimal lesion formation, such as reperfusion injury in bypass grafts or organ transplants (Waltenberger et al., 1996), inflammation, infection (Koskinen et al., 1993) and hyperlipidemia (Räisänen-Sokolowski et al., 1994), among other causes. The similarity in lesion morphlogy in transplant arteriosclerosis, the fibrous atherosclerotic plaques and restenotic arteries leads to the inevitable assumption that the pathogenesis of these differentially primed disorders is the same. The current hypothesis is that atherosclerotic and restenotic lesions constitute mainly of macrophages, T-cells and myofibroblasts or SMC derived from adjacent tissues and circulation (Libby et al., 1992; Ross, 1993).

2. Experimental models for studying vasculoproliferative disorders

Rat or rabbit aortic or carotid ballooning injury are widely used models to study the mechanisms of SMC migration and proliferation in arteriosclerosis and restenosis. These rodent models, however, are not to be applied directly to human settings of restenosis, where various risk factors, such as individual susceptibility, gender, and other factors are to be taken into account. Also, the protocol in human angioplasty always involves antiplatelet/anticoagulant therapy, which in rats are usually not applied. However, these rodent models allow us to study separately the cellular events after endothelial injury in an *in vivo* environment, and allow us to exclude some of the sources of error possible in *in vitro* models, such as cell differentiation/dedifferentiation in vitro. Several agents have proved effective in animal models, such as angiotensin converting enzyme inhibitors (Powell et al., 1989), angiotensin or its receptor inhibitors (Forney Prescott et al., 1991) and heparin (Rogers et al., 1996), have failed to be effective in human studies (The MERCATOR Study Group, 1994; Ellis et al., 1989). Some results, however, suggest that data derived from rodent models can be applicable to human settings (Serruys et al., 1997): the efficacy of inhibition of neointimal formation with PDGF inhibitors is mainly derived from studies made on rodents, and it

has been shown that Trapidil, a specific PDGF inhibitor inhibits restenosis by as much as 50% in humans (Maresta et al., 1994).

3. Neointimal smooth muscle cells

Evidently, rat models of arterioslerosis or restenosis differ from human lesions in many respects: rats are very resistant to hypercholesterolaemia, so the lesions lack oxidised low-density lipoproteins and foam cells. Mice defective in apolipoprotein E, are spontaneously hypercholaesterolaemic and develop lesions rich in T cells and activated macrophages (Lichtman et al., 1996; Zhou et al., 1996). In rodent models, after single ballooning injury, the amount of inflammatory cell infiltrates and adhesion molecule expression is not prominent with the possible exception of hyperlipidemic rabbits (Tanaka et al., 1993). However, when reinjury is performed on a preformed neointima, the lesion becomes rich in macrophages and T cells, suggesting that the response-to-injury to a preformed neointimal layer is different from injury to an intact artery. A functional difference in the first vs. the second injury has also been shown: antibodies to bFGF and heparin, both well known inhibitors of proliferation after the first injury, do not have an effect on proliferation after the second ballooning injury (Koyama and Reidy, 1997), nor in human restenosis (see above and Mak and Topol, 1999). Furthermore, we show in this study, that selective inhibition of PDGF-R is noneffective in reducing proliferation after endothelial injury to intact rat aortas (first injury), whereas after a second injury to preformed neointima, the same compound is highly efficient in inhibiting neointimal proliferation.

Studies made with cloned SMC in vitro and cultured neointimal cells indicate that cells of neointimal phenotype express PDGF receptors and proteins differentially from resting or contractile phenotype SMC (Lemire et al., 1994). Neointimal SMC resemble SMC isolated from young rats: They represent a more primitive, dedifferentiated phenotype expressing the PDGF-B gene but hardly any PDGF-Rα (Lemire et al., 1994). Two recent studies show that PDGF selectively induces intimal proliferation without having any effect on medial cell proliferation (Pompili et al, 1995; Sirois et al., 1997), suggesting that the response of neointimal SMC to injury differs from the response of medial SMC. Adding further evidence to the functional difference between injured medial versus neointimal cells, we show in this study, that after repeated injury to rat aorta, the enhanced proliferative response was linked to increased PDGF-AA, and PDGF-Rß expression, and the inhibition of the PDGF-R had little effect on medial or

neointimal SMC proliferation, but a pronounced effect on the proliferation of neointimal cells after reinjury.

There is currently little information on the functional role of adhesion molecules in SMC. It is classically thought that their primary role is to mediate leukocyte adhesion to endothelial and SMC preceding local immune reactions (Libby et al., 1992). However, more recent data suggests that these endothelial adhesion molecules are also expressed in developmental stages of blood vessels (Couffinhal et al., 1994). Results presented in this study show that the VCAM-1 adhesion molecule is expressed in SMC during and shortly after proliferation has occurred. Very high levels of VCAM-1 were seen in SMC 3 d after injury in the medial cells, when high levels of proliferating cells are seen, but practically no inflammatory cells were detected in the media during or following peak VCAM-1 expression. Why would VCAM-1 be expressed in the media if its expression is not linked with increased leukocyte adhesion? VCAM-1 is a marker for a dedifferentiated SMC phenotype and may act as a receptor to extracellular matrix components mediating cell adhesion; an absolute requirement for the migration and proliferation of mesenchymal cells. Adhesion molecule P-selectin was also found to be upregulated in regenerating endothelial cells. This upregulation may be linked to a more efficient reqruitment of inflammatory cells to the neointima. P-selectin immunoreactivity was also seen in platelets adherent to the internal elastic lamina after balloon injury, possibly increasing leukocyte rolling/adhesion to sites of injury.

Recent studies have shown that endothelial cell progenitors can be isolated from the peripheral blood, and that these cells contribute to angiogenesis (Asahara et al., 1997). Others have shown that bone-marrow derived myogenic progenitor cells contribute to the regeneration of striated muscle (Ferrari et al., 1998). Similarly, neointimal SMC could originate from multipotent bone marrow stem cells via the circulation, which could explain the phenotypic and functional differences in neointimal and medial SMC.

4. Inflammation in arteriosclerosis and restenosis

Monocytes and macrophages have been known to be present in arteriosclerotic lesions both in classical atherosclerosis (Ross et al., 1984) and in transplant arteriosclerosis (Rose and Uys, 1990). In vasculitis syndromes as well as in transplants undergoing chronic rejection the correlation between inflammation and neointimal lesion formation is a well-known late complication (Häyry et al., 1993; Somer, 1993). The role of

inflammation, however, in lesions of classical atherosclerosis or restenosis may well have been underestimated so far. Recently, epidemiological as well as experimental evidence has been accumulating on the role of inflammation in atherosclerosis:

First, there has been epidemiological evidence on the association of chlamydia pneumoniae (Saikku et al., 1988) and cytomegalovirus (Koskinen et al., 1993) to atherosclerosis. Second, some of these micro-organisms have been detected in neointimal lesions both in atherosclerotic (Kuo et al., 1993; Chiu et al., 1997) and restenotic (Speir et al., 1994) lesions. Third, antibiotic treatment has in some studies shown to decrease complications to acute myocardial infarction, suggesting an infectious epidemiology to atherosclerosis (Gupta et al., 1997). It would be of interest to find out, whether antibiotic or immunosuppressive treatment decreases the morbidity to atherosclerosis by studying e.g., the incidence of coronary artery disease in other than heart transplant patients.

In this study, we show that the level of inflammatory cells increases after injury to preformed neointima and hypothesize that expression of adhesion molecules in the neointimal layer may lead to more efficient recruitment of leukocytes when this preformed neointima is injured again.

5. Therapeutic strategies for vasculoproliferative disorders

Prolonged cold preservation is a risk factor for transplant arteriosclerosis. This is believed to be due to the generation of toxic oxygen radicals. Independent of cold-preservation/reperfusion -related free radical formation, oxidative processes are believed to enhance atherosclerosis, although some controversy exists, whether antioxidants prevent atherosclerosis (reviewed by Steinberg, 1995; Berliner and Heinecke, 1996). We attempted to decrease cold-ischemia -induced injury in syngeneic rat aortic transplants by perioperative administration of rh-SOD. No effect in atherosclerotic lesion formation in the transplanted grafts after 1-6 months was seen, whereas clinical evidence upon the beneficial effects do exist (Land et al., 1994).

More than half of neointimal cells after a two-week observation time have migrated there rather than derived from cell division (Clowes and Schwartz, 1985). So far, few specific inhibitors of migration exist, and studies showing the effect of inhibition of migration to final lesion formation are not many (Bendeck et al., 1996; Zempo et al.,

1996). The mechanisms of migration and proliferation overlap in many respects, and the same intracellular signaling cascades for migration and proliferation are used in cells. The results presented here suggest that the mere inhibition of migration may suffice in order to - at least partially - inhibit neointimal lesion formation. However, practically nothing is known about the inhibition of migration in restenotic lesion formation or in the reinjury models.

Although recent evidence suggests that proliferating cells are infrequently seen in atherectomy specimens (O´Brien et al., 1993b) several histopathological findings from restenosed arteries support a role for intimal proliferation in vessel occlusion (Essed et al., 1983, Austin et al., 1985; Nobuyoshi et al, 1991). Some studies also suggest that adventitial fibroblasts contribute to lesion formation after extensive ballooning injury (Shi et al., 1996ab; Scott et al., 1996). This study among others (Koyama and Reidy, 1997; Stadius et al., 1996) shows that in rodents after reinjury of intima, proliferative response is more rapidly downregulated and proliferating cells are not sustainedly seen as they are after the first injury. It would, therefore, seem plausible that in human lesions, high levels of proliferating cells would not be seen in mature restenotic lesions. Controversially, in reinjury models, as well as in human restenosis neointimal size has no correlation to luminal narrowing (Courtman et al, 1988; Andersen et al., 1996), suggesting that the remodelling of blood vessels accounts for a compensatory enlargement of the vessel lumen. Furthermore, increase in neointimal mass is not correlated with an increase in cell numbers (Koyama and Reidy, 1988). Nevertheless, after remodelling capacities of the blood vessel are lost, as in fibrosed human coronary arteries subjected to angioplasty, neointimal proliferation and lesion formation evidently leads to occlusion of artery and tissue hypoxia. Therefore, antiproliferative therapy remains a plausible target for human restenosis.

6. Candidate drugs

It has been suggested that various proproliferative factors for SMC act via the upreglation of IGF-1R and thus create an autocrine cycle that finally triggers cell cycle progression to mitosis (Baserga, 1993). Findings made principally by Delafontaine and colleagues using antisense oligonucleotides or antisera to the IGF-1R show that growth induced by PDGF, angiotensin and thrombin at least are mediated by the IGF-1R (Ververis et al., 1993; Delafontaine et al., 1996). These studies indicate that an IGF-1/IGF-1R autocrine cycle is required for optimal growth of vascular SMC. Based on

these findings, and on the upregulation of IGF-1R during SMC proliferation we hypothezised that the D-peptide analog of IGF-1, JB3, could interfere with the proliferative response to SMC after carotid ballooning injury, which, indeed, was the case. The effect of inhibition of the IGF-1R has not previously been shown in vivo in vascular proliferation. We were, however, unable to show that lesion size could actually be affected by the inhibition of the IGF-1R in the rat carotid artery. This can partially be due to the nature of the D-peptide analog as a competitive inhibitor, it may not inhibit completely the activeation of the IGF-1R. Also, our findings suggest that inhibition in proliferation may not sufffice for efficient inhibition of lesion formation.

Since migration of cells in as much as 50% is believed to contribute to neointimal lesion formation (Clowes et al., 1983; Clowes and Swartz, 1985), the inhibition of migration might therefore also be a requirement for optimal reduction in lesion formation after ballooning injury. PDGF, on the other hand, has been shown to induce lesion formation with a minor effect on cell proliferation in rat carotid artery (Ferns et al., 1991). Therefore, inhibitor of the PDGF-R would in all likelihood inhibit migration but could also affect proliferation possiby indirectly acting via the IGF-1R.

Highly selective receptor tyrosine kinase inhibitors have been developed (Levitzki and Gazit, 1995; Levitzki, 1996). Nonselective tyrphostins have been shown to be inhibitory to lesion formation in rat carotid artery (Golomb et al., 1996). Tyrphostins selective for the PDGF-R have been shown to inhibit the formation of neointima in a porcine model of restenosis when applied locally (Banai et al., 1998) supporting our concept of PDGF-blockade as a treatment strategy for restenosis. Tyrphostin inhibitors for the IGF-1R that have 100 times lower affinity to the highly homologous insulin receptor have been developed 1 data strongly indicates that the development of even more selective receptor tyrosine kinase inhibitors will soon be available for experimental animal and human studies. Specific inhibitors of the IGF-1R would be extremely interesting to test in models of vascular proliferation, since e.g. peptide analogs have the disadvantage of being difficult to produce and their administration as a continuous infusion is suboptimal.

SIGNIFICANCE OF FINDINGS

This study supports the accumulating amount of evidence indicating that neointimal SMC differ from resting, medial SMC in their ability to proliferate in response to injury, in their activation state and response to PDGF-R activation. Also, this study is the first one to show that neointimal cells are more resistant to programmed cell death than medial SMC - a finding which may also be helpful in understanding the pathogenesis of restenosis. Inhibition of IGF-1 or PDGF receptors can be used as therapeutic strategies in the inhibition of vasculoproliferative disorders such as restenosis after coronary angioplasty. More detailed studies in large animal models or human trials will eventually show whether this approach could also be used in treatment of human restenosis.

SUMMARY

The thickening of the innermost layer of arteries, the neointima, and remodelling of the vascular wall are the principal pathogeneic mechanisms underlying atherosclerosis, restenosis and transplant arteriosclerosis. Injury to arteries - regardless of its cause - leads to proliferation of SMC in the medial (muscular) layer, and the subsequent migration of these cells into the neointima, where they start by proliferating to form the neointimal layer.

The first objective of this study was to investigate the formation of arteriosclerotic lesions using experimental animal and *in vitro* models, as well as to develop more suitable models for studying the mechanisms of restenosis.The second objective in this study was to prevent pharmacologically neointimal formation and thickening of arteries using specific inhibitors of the IGF-1 and PDGF receptors. Three rat models were used for studying neointimal lesion formation: cold-ischemia-induced arteriosclerosis, carotid artery ballooning injury, and ballooning reinjury of the rat aorta. Quantitative studies on SMC migration and proliferation were performed using SMC cultures.

The results showed that all experimental models induced severe arteriosclerosis-like changes, with the common feature of SMC-rich neointimal lesions. We demonstrated that in cold-ischemia -induced arteriosclerosis, and after ballooning injury of the rat carotid artery, upregulation of several peptide growth factors was observed during lesion formation. The results indicated that the local production of growth factors may contribute to neointimal formation. When ballooning injury was performed on a pre-existing neointimal lesion, as is done in human angioplasty operations, we were able to show that the proliferative and apoptotic response, and the cellular composition differed significantly from a lesion induced by a single ballooning injury to rat arteries.

We then studied the effect of blocking some of these upregulated growth factors in lesion formation in rat carotid artery after injury. Using a stable D-peptidic analog of IGF-1, we were able to inhibit neointimal SMC proliferation in rat carotid artery. Using a selective inhibitor of the PDGF-R an effect was seen primarily in SMC migration and at the increase in lesion size at the end point of observation. Finally, we were able to inhibit the enhanced proliferative response after ballooning reinjury by a specific inhibitor of the PDGF-R.

The results of this study indicate that IGF-1 and PDGF are major regulators of SMC migration and proliferation *in vivo*. We also show that neointimal SMC are more resistant to programmed cell death than medial SMC, and respond differentially to the inhibition of PDGF-R. The findings may be useful in the understanding of the pathogenesis of restenosis after angioplasty. Inhibition of PDGF-R can have clinical applications in the preventive therapy for restenosis.

YHTEENVETO (FINNISH SUMMARY)

Valtimoiden sisäseinämän paksuuntuminen aiheuttaa verisuonen ahtautumisen valtimoiden kovettumataudin varhaisessa vaiheessa ja sepelvaltimoiden pallolaajennuksen jälkeisessä uudelleenahtautumisessa, restenoosissa. Restenoosi on yleinen komplikaatio onnistuneen pallolaajennuksen jälkeen; n. 30-40% pallolaajennetuista sepelvaltimoista ahtautuu uudelleen puolen vuoden sisällä operaatiosta.

Verisuonten sisäpintaa verhoavan endoteelisolukon vaurio johtaa yleensä sileän lihassolukon jakautumiseen valtimoiden keski- eli lihaskerroksessa. Osa näistä jakautuneista lihassoluista migroituu sisäseinämän puolelle ja jakautuu edelleen ahtauttaen suonta. Endoteelivaurion aiheuttaja voi olla mekaaninen (pallolaajennuskatetri), immunologinen (elinsiirrännäiseen kohdistuva hyljintäreaktio) tai oksidaation aiheuttama (reperfuusiovaurio tai hapettuneen LDL:N kertyminen verisuoneen). Riippumatta endoteelivaurion laadusta, valtimoiden ahtautumiseen johtavat sileälihassoluvasteiden mekanismit ovat hyvin pitkälle samankaltaisia.

Väitöskirjatyön tarkoituksena oli tutkia kokeellissa eläinmalleissa tapahtuvaa verisuonivaurion jälkeistä jakautumis- ja migraatiovastetta sekä kehittää restenoosin mekanismien tutkimiseen soveltuva koe-eläinmalli. Lisätavoitteena oli vähentää verisuonen sisäseinämän kasvua farmakologisesti estämällä insuliinin kaltaisen kasvutekijän (IGF-1) ja verihiutaleperäisen kasvutekijän (PDGF, platelet-derived growth factor) toiminta.

Tutkimusmenetelminä kaytettiin kolmea erilaista kokeellista rottamallia: kylmäiskeamian indusoimaa ateroskleroosia, rotan kaulavaltimon pallolaajennuksen aiheuttamaa ateroskleroosia ja rotan aortan kaksoisvauriomallia (restenoosin mallina). Soluviljelmässä tutkittiin lääkeaineiden vaikutusta sileän lihassolukon migraatioon ja jakautumiseen ennen lääkkeiden testaamista koe-eläimessä.

Tutkimustulokset osoittavat, että kaikissa kokeellisissa malleissa verisuoniin kehittyy ensisijaisesti sileästä lihassolukosta koostuva sisäseinämän paksuuntuma. Lisäksi tulokset osoittivat, että verisuonen seinämän kasvutekijätuotanto lisääntyy solujen jakautuessa ja vaurion kehityessä. Kasvutekijöillä oli vaikutusta ahtauman muodostumiseen: estämällä IGF-1:n sitoutuminen reseptoriinsa sen D-peptidianalogilla

(kompetitiivinen inhibitio), sileän lihassolukon jakautumisvaste estyi n. 50 %:sti. Vaikutus valtimon sisäseinämän paksuuntumiseen oli kuitenkin vähäinen. Estämällä PDGF-reseptorin aktivoivan tyrosiinikinaasin toiminta valtimon sileälihassolukon migraatio estyi n. 90%:lla ja sisäseinämän paksuuntuminen estyi 40%:lla.

Valtimoiden pallolaajennuksen jälkeisen uudelleenhtautumisen, restenoosin tutkimiseksi kehitimme rotan aortan kaksoisvauriomallin. Tällä mallilla osoitettiin, että jakautumisvaste vaurioitettaessa jo ahtautunutta suonta eroaa huomattavasti vauriosta terveeseen suoneen: jakautumisvaste on nopeampi ja voimakkaampi. Osoitimme myös, että sisäseinämän solut ovat resistenttejä vaurion aiheuttamalle ohjelmoidulle solukuolemalle verrattaessa lihassolukerroksen soluihin. Nopea sileän lihassolukon jakautumisvaste ja ahtauman muodostaminen on estettävissä estämällä PDGF-reseptorin toiminta.

Väitöskirjassa osoitettiin, että valtimon sisäseinämän solut ovat resistentimpiä ohjelmoidulle solukuolemalle kuin lihassolukerroksen solut, mikä selittää pallolaajennuksen jälkeisen valtimoiden uudelleenahtautumisen patogeneesiä. IGF-1 ja PDGF -kasvutekijäreseptorit ovat merkittäviä sileän lihassolukon kasvun säätelijöitä valtimoissa *in vivo*. PDGF-reseptorien estäjillä saattaa tulevaisuudessa olla huomattava kliininen ja taloudellinen merkitys restenoosin ennaltaehkäisevässä hoidossa.

SAMMANDRAG (SWEDISH SUMMARY)

Skada mot endotelcellagret, dvs. det innersta cellagret i artärer leder till blodkärlsförtjockning. Förtjockningen beror på att glatta muskelceller (GMC) från blodkärlets mellersta del (tunica media) migrerar till den inre delen (tunica intima) och prolifererar däri. Migration och proliferation av glatt muskelvävnad är den principiella mekanismen bakom restenos, dvs. en upprepad förträngning av koronärartärer, som sker efter koronärangioplasti i 50-80% av fallen. Oberoende av endotelskadans orsak, som kan vara mekanisk, (angioplastikateters in koronarangioplasti), immunologiskt (rejektion i transplanterade organ, eller en frigörelse av fria syreradikaler (reperfusionskada), är mekanismen bakom GMC migrationen och proliferationen densamma. Det har visats att att det finns flera tillväxtfaktorer för GMC, av vilka är en av de mest undersökta insulin-liknande tillväxtfaktorn IGF-1, och blodtillväxtfaktorn, PDGF (platelet-derived growth factor). Tillsvidare finns det inga effektiva läkemedel för att förebygga restenos eller transplantationsateroskleros.

Avsikten med denna undersökning var att studera blodkärlsförtjockning i försöksdjurmodeller. Vi ville också utveckla en lämpligare försöksdjursmodell för att studera restenos. Avsikten var också att farmakologiskt förebygga åderförtjockning genom att hämma IGF-1 och PDGF -receptorer hos försöksdjur.

Ballongangioplasti är mycket använd i försöksdjursmodeller för att studera okklusion av artärer. En ballongskada skapas mot råttans halsartär eller aorta. I undersökningen har tre olika metoder använts för att studera ocklusion i artärer efter endotelskada: kalliskemi-inducerad ateroskleros, ballongskada mot råttans halsartär eller aorta, och dubbelballongskada mot råttans aorta. Migration och proliferation av glatta muskelceller och inverkan av läkemedel studerades i dessa modeller samt i cellkulturer.

Resultaten visade, att i alla dessa modeller består de förtjockade artärerna av GMC. Vi visade ochså, att att när cellerna delar sig, ökar tillväxtfaktorproduktionen i blodkärlen, vilket tyder på att tillväxtfaktorerna har betydelse i åderhyperplasi. Vi visade även, att när man hämmar IGF-1 genom att binda receptoren med IGF-1 D-peptidanalog, är det möjligt att minska GMC proliferation med 50%. Inverkan på åderhyperplasi var mindre. Genom att hämma PDGF-receptoraktivationen med en tyrosinkinashämmare, var det möjligt att minska migration av GMC med 90%, och åderhyperplasi med 40%.

En av de största nackdelar i ballongskademodellerna är, att skadan förorsakas på en intakt artär. Som terapeutisk åtjärd görs interventionerna däremot alltid på en förtjockad eller sklerotiskt artär. Genom att skada råttans aorta två gånger med två veckors mellanrum har vi kunnat påvisa, att den proliferativa responsen mot en upprepad ballongskada inträffar betydligt snabbare och är mera intensiv jämfört med responsen vid skada mot en intakt artär. Vi visade också, att GMC i intima är mera resistenta för apoptos efter ballongskada, än vad GMC i media är.

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