Helsinki Biomedical Graduate School

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VASCULAR BIOLOGY OF
ESTROGEN RECEPTORS ALPHA AND BETA
– AN EXPERIMENTAL APPROACH TO
RESTENOSIS AND TRANSPLANT ARTERIOSCLEROSIS

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ACADEMIC DISSERTATION

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To Lauri and Juho

CONTENTS

ORIGINAL PUBLICATIONS

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

I   Mäkelä S, Savolainen H, Aavik E, Myllärniemi M, Strauss L, Taskinen E, Gustafsson JÅ, Häyry P. Differentiation between vasculoprotective and uterotrophic effects of estrogen via ligands with different binding affinities to estrogen receptor α and β. Proc Natl Acad Sci USA. 1999; 96: 7077-7082.

II   Savolainen H, Frösen J, Petrov L, Aavik E, Häyry P. Expression of estrogen receptor subtypes α and β in acute and chronic cardiac allograft vasculopathy. J Heart and Lung Transplant. 2001; 20: 1252-64.

III   Savolainen-Peltonen H, Luoto NM, Kangas L, Häyry P. Selective estrogen receptor modulators prevent neointima formation after vascular injury. Molecular and Cellular Endocrinology, *in press*.

IV   Savolainen-Peltonen H, Loubtchenkov M, Petrov L, Delafontaine P, Häyry P. Estrogen regulates insulin-like growth factor 1, platelet-derived growth factor-A and -B, and their receptors in the vascular wall. Transplantation. 2004; 77: 35-42.

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ABBREVIATIONS

ACE	angiotensin converting enzyme
AF	activation fuction
AT	angiotensin
cAMP	cyclic adenosine monophosphate
CEE	conjugated equine estrogen
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CMV	cytomegalovirus
CRP	C-reactive protein
CsA	cyclosporine A
DA	Dark Agouti rat strain
DBD	DNA binding domain
DMSO	dimethyl sulphoxide
E1	estrone
E2	(17β-)estradiol
E3	estriol
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response element
ET	endothelin
HLA	human leukocyte antigen
HRT	hormone replacement therapy
hsp	heat shock protein
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MHC	major histocompatibility antigen
MPA	medroxyprogesterone acetate
NO	nitric oxide
NOS	nitric oxide synthase
NR	nuclear receptor
PAI	plasminogen activator inhibitor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PTCA	percutaneous transluminal coronary angioplasty
SERM	selective estrogen receptor modulator
SHBG	sex-hormone binding globulin
SMC	smooth muscle cell
TGF	transforming growth factor
TNF	tumor necrosis factor
TPA	tissue-type plasminogen activator
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
WF	Wistar Furth rat strain

ABSTRACT

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) and allograft arteriosclerosis after organ transplantation limit the long-term survival of operated patients. The diseases are characterized by accumulation of smooth muscle cell (SMC)-like cells to the luminal side of the affected artery, their proliferation, migration, and extracellular matrix formation, which leads to the development of neointimal hyperplasia and constrictive vascular remodelling. Recent studies indicate that the proliferating neointimal SMCs derive from multipotent somatic stem cells (vascular progenitors), which are able to differentiate into vascular cells.

The vasculoprotective properties of estrogens have been demonstrated in epidemiological and experimental studies, although some recent studies show contradictory evidence. Estrogen therapy is associated with increased risk for endometrial cancer, and may promote the growth of breast cancer, which limits its use in vasculoprotection. In addition to the classical estrogen receptor (ER) α, a novel ER, ERβ, was discovered in 1996, which greatly improved our understanding of estrogen's signalling in different tissues during health and disease. The roles of the two receptors in the vascular wall are not completely understood.

The aim of this study was to clarify the expression and regulation of the two ERs in different vasculoproliferative disorders, and to identify potential mechanisms behind estrogen's vasculoprotective action, using rat denudation injury and cardiac transplantation models. The study shows substantial upregulation of ERβ in the vascular wall after mechanical and alloimmune injury, whereas ERα remains expressed at a low level only. ERβ is localized in the neointimal and medial SMCs of the arteries as well as in the veins, myocardium, and inflammatory cells of cardiac allografts, which suggests that these cells are targets for estrogen. Moreover, estrogen's vasculoprotective properties in the injured vascular wall can be differentiated from its uterotrophic action with a ligand that shows higher binding affinity for ERβ. Thus, preferential targeting to ERβ might be useful when aiming to develop vasculoselective estrogens. Selective estrogen receptor modulators (SERM), which act as estrogen agonists in some tissues and are anti-estrogenic in others, preserve estrogen's inhibitory effect on denudation injury-induced neointimal thickening by inhibiting vascular SMC-like cell proliferation and migration, *in vivo* and *in vitro*, and by enhancing reendothelialization. The impact of different SERMs on neointima formation varies depending on the timing of the treatment, where early timing appears to be particularly important.

Finally, estradiol treatment after denudation injury downregulates the expression of insulin-like growth factor 1, platelet-derived growth factor, and platelet-derived growth factor receptor α mRNA and protein, which is probably one of the mechanisms behind estrogen's vasculoprotective actions.

INTRODUCTION

Cardiovascular disease, caused by atherosclerosis, is the major cause of death in the Western world. Percutaneous transluminal coronary angioplasty (PTCA) is commonly used to relieve the symptoms of atherosclerosis. It is, however, complicated by restenosis in 30% to 50% of the patients (Holmes et al. 1984, Gruentzig et al. 1987, Nobuyoshi et al. 1988). Allograft arteriosclerosis is a major limitation to the long-term success rate in transplantation, and furthermore, about 50% of kidney grafts are lost to death with function - mainly for cardiovascular reasons (Lindholm et al. 1995). The pathogenesis of these vasculoproliferative disorders is still incompletely known and there are no effective therapies for their prevention.

The development of atherosclerosis, restenosis, and allograft arteriosclerosis has been defined as a response to immunological, mechanical, or infectious injury to the arterial wall (Hayry et al. 1993, Ross 1993). Peculiar to the disorders is an inflammatory response followed by an influx of smooth muscle cell (SMC)-like cells to the luminal side of the affected artery; their proliferation, migration, and extracellular matrix formation, which leads to the development of neointimal hyperplasia and vascular remodelling. Recent studies suggest that the proliferating neointimal SMCs derive from multipotent somatic stem cells (vascular progenitors), which are able to differentiate into vascular cells (Hillebrands et al. 2000, Saiura et al. 2001). Peptide growth factors have been recognized as important regulators of SMC kinetics in the vascular wall (Cercek et al. 1991).

The vasculoprotective properties of estrogens have been widely demonstrated in epidemiological and experimental studies (Farhat et al. 1996b, Mendelsohn and Karas 1999), although some recent prospective studies show conflicting data (Hulley et al. 1998, Rossouw et al. 2002). It was difficult to understand how estrogen therapy can - at the same time - induce proliferation in the endometrium and breast while being anti-proliferative in the cardiovascular system. A new era in the understanding of estrogen action began in 1996 with the discovery of a novel estrogen receptor (ER), ERβ, in addition to the classical ERα (Kuiper et al. 1996, Mosselman et al. 1996). Each of these receptors has distinct tissue distribution and regulation, which enables the development of tissue-selective estrogens. Although knock-out mouse studies suggest that either of the two ERs may mediate estrogen's protective effects on the vascular wall (Iafrati et al. 1997, Karas et al. 1999, Karas et al. 2001), their contribution to the inhibition of neointimal thickening is still incompletely understood.

The purpose of this study was to investigate the expression and regulation of the two ERs in the mechanical or immunological injury-induced development of neointimal hyperplasia, to try to differentiate estrogen's beneficial vasculoprotective properties from its gender-related side-effects with ER subtype-selective estrogen, and to try to elucidate the mechanisms behind estrogen's vasculoprotective action.

REVIEW OF THE LITERATURE

1. Vasculoproliferative disorders

1.1. Atherosclerosis

Atherosclerosis is defined as a chronic inflammatory fibroproliferative disorder which causes narrowing of the affected arteries and produces ischemia of tissues perfused through these arteries (Ross 1986, Ross 1993, Ross 1999). It is the major cause of cerebral and myocardial infarction, ischemia of the intestines and lower extremities, and accounts for more than half of deaths in the Western world.

The early lesions in atherosclerosis consist of cholesterol-filled macrophages, 'foam cells', in the subendothelium of the arterial wall (Ross 1993, Ross 1999). These fatty streaks may be found in the aorta already during the first decade of life, and they are precursors to the more advanced lesions, called 'fibrous lesions'. Characteristic to such lesions is a 'fibrous cap' consisting of SMC and extracellular matrix (ECM), and a lipid-rich necrotic core. Advanced lesions may also become calcified and ulcerated, or complicated with hemorrhage. The most important clinical complication in an atherosclerotic artery is acute occlusion due to the formation of a thrombus, usually caused by a rupture of the plaque, resulting in myocardial infarction or stroke.

Atherosclerosis is a multifactorial disease. Well-recognized risk factors associated with the disease include age, male sex, familial predisposition, hyperlipidemia, hypertension, diabetes, smoking, and lack of exercise. More recently, other possible risk factors have been identified, such as elevated levels of C-reactive protein (CRP), homocysteine, fibrinogen, plasminogen activator inhibitor (PAI) I, and apolipoprotein(a) (Grainger et al. 1994, Thompson et al. 1995, Graham et al. 1997, Nygard et al. 1997). Also, several studies indicate that infectious agents like *Chlamydia pneumoniae* (Saikku et al. 1988, Kuo et al. 1993) and cytomegalovirus (CMV) (Sorlie et al. 1994, Nieto et al. 1996) are associated with an increased risk for atherosclerosis.

Changes in lifestyle are essential for the prevention of atherosclerosis. Moreover, clinical trials have demonstrated the benefits of lipid-lowering therapies (Shepherd et al. 1995, Sacks et al. 1996, Downs et al. 1998) and blood pressure reduction (Hansson et al. 1998) in primary and

secondary prevention of cardiovascular disease. Although several pilot studies have shown positive effects of antibiotic treatment against chlamydia pneumoniae in the secondary prevention of cardiovascular events (Gupta et al. 1997, Gurfinkel et al. 1997), no large randomized trials have so far confirmed those findings (Cercek et al. 2003, O'Connor et al. 2003).

1.2. Restenosis

Percutaneous transluminal coronary angioplasty (PTCA), first performed in 1977 (Gruntzig et al. 1979), has become a well-established technique for myocardial revascularization to relieve symptoms of coronary heart disease (CHD). However, restenosis, defined as reocclusion of the operated artery, remains a remarkable problem after the operation. Restenosis occurs in about 30%-50% of the patients within 3 to 6 months after successful intervention (Holmes et al. 1984, Gruentzig et al. 1987, Nobuyoshi et al. 1988), and often leads to reoperation.

Characteristic to restenosis is the accumulation of vascular SMC to the luminal side of the artery, their proliferation and migration, and extracellular matrix formation, which results in neointimal hyperplasia (Libby et al. 1992, Haudenschild 1993, Casscells et al. 1994). Negative remodelling, or constriction of the artery, also plays a crucial role in the process (Lafont et al. 1995, Mintz et al. 1996). Reconstitution of the endothelium limits the growth of the neointima with time (Fishman et al. 1975, Clowes et al. 1978, Haudenschild and Schwartz 1979).

It is difficult to predict reocclusion of the artery from clinical variables and current data on the related risk factors are inconsistent. Certain patient-related risk factors, such as age, diabetes, hypertension, severe angina, genetic predisposition, and elevated fibrinogen and homocysteine levels, have, however, been associated with restenosis (Rensing et al. 1993, Weintraub et al. 1993, Montalescot et al. 1995, van Bockxmeer et al. 1995, Schnyder et al. 2002). The most important predictive lesion characteristics are small vessel diameter, long lesion length, and very small lumen diameter before and after the operation (Rensing et al. 1993, Foley et al. 1994)

There is currently no effective treatment for restenosis. Several pharmacological approaches, e.g., with angiotensin converting enzyme (ACE) inhibitors, β-blockers, and methylprednisolone, have been attempted but none of them has been effective in preventing

the disease (Pepine et al. 1990, Faxon 1995, Serruys et al. 2000). By the year 2002, of the 37 compounds tested, only 4 demonstrated efficacy in randomized placebo-controlled studies (Table 1). Metal intracoronary stents, which are placed at the point of the atherosclerotic occlusion during PTCA, were introduced during the 1990's (Serruys et al. 1991, Fischman et al. 1994). The stent maintains the vessel lumen diameter, and thus prevents elastic recoil and negative remodelling; however, excessive neointimal hyperplasia, or in-stent restenosis (Virmani and Farb 1999), remains a significant problem affecting 10%-30% of patients with stent. Drug-eluting stents, which deliver the medication directly to the site of injury, have so far proven efficacious with minimal in-stent restenosis during a 6 to 12 month follow-up (Sousa et al. 2001a, Sousa et al. 2001b, Morice et al. 2002, Grube et al. 2003). Their long-term efficacy and safety are still to be investigated.

Table 1. Clinical experience in the prevention of restenosis

Compound	Target	Efficacy	References
Linsidomine (PTCA)	NO donor	45% vs. 38% (p=0.03)*	Lablanche et al. 1997
Trapidil (PTCA)	PDGF antagonist	40% vs. 21% (p=0.01)*	Okamoto et al. 1992, Maresta et al. 1994
Angiopeptin (PTCA)	Somatostatin receptors 2, 3, 5	40% vs. 12% (p=0.003)*	Eriksen et al. 1995
Rapamycin (stent)	mTOR	27% vs. 0% (p=0.001)*	Morice et al. 2002

* 6-month or ** 3-month endoscopic follow-up

1.3. Transplant arteriosclerosis and accelerated recipient atherosclerosis

Although the development of new immunosuppressive drugs has dramatically improved the short-term success rate in transplantation, long-term survival (after one post-operative year) has not improved as much, and chronic allograft rejection remains the leading cause of late graft failure. Furthermore, in renal transplantation, about half of the late failures are due to recipient death with a functioning graft, mainly because of cardiovascular reasons (accelerated atherosclerosis) (Lindholm et al. 1995, Kasiske et al. 1996).

Chronic rejection is histologically characterized as persistent perivascular inflammation, fibrosis, and arteriosclerosis of all intra-graft arteries to the level of arterioli. Unlike in common atherosclerosis, the vascular lesions in transplant arteriosclerosis are often diffuse and concentric; not focal and eccentric. They are composed mainly of α-actin expressing SMC and extracellular matrix; calcifications are usually not seen (Johnson et al. 1991). These common histological changes in all transplants are accompanied with organ-specific

alterations, such as basement membrane thickening, glomerular sclerosis, and tubular atrophy in the kidney (Isoniemi et al. 1992).

The etiology of chronic rejection is multifactorial. Several factors have been shown to influence the long-term outcome of the graft, and they are usually divided into alloantigen-related, alloantigen-unrelated, and infectious factors. Alloantigen-related factors include histoincompatibility, insufficient immunosuppression, presence of anti-HLA antibodies ('pre-sensitisation'), and acute rejection (Suciu-Foca et al. 1991, Opelz 1992, Almond et al. 1993, Basadonna et al. 1993, Yilmaz and Hayry 1993). Alloantigen-unrelated factors include very young or very old donor age, ischemia-reperfusion injury, hyperlipidemia, hypertension, diabetes, and donor-recipient size-incompatibility (Cheigh et al. 1989, Cho et al. 1989, Lim and Terasaki 1991, Yuge and Cecka 1991, Heemann et al. 1994, Isoniemi et al. 1994, Dimeny et al. 1995). Finally, infectious factors, such as CMV infection, have been associated with the development of graft failure, allograft arteriosclerosis, and death after transplantation (O'Grady et al. 1988, Grattan et al. 1989, McDonald et al. 1989).

At the moment, there is no specific therapy for chronic rejection. Only preventive measures are available and their aim is to intervene with the potential risk factors in the disease process. As mentioned above, the development of immunosuppressive drugs, especially the introduction of cyclosporine in the late 70's (Calne et al. 1978), dramatically decreased the incidence of acute rejection and improved the early survival after transplantation. In the 1990's, new drugs have been developed, including mycophenolate mofetil, tacrolimus, sirolimus, and everolimus. Their long-term effects in humans are still under investigation. However, everolimus has been shown to significantly reduce transplant arteriosclerosis in cardiac allograft recipients (Eisen et al. 2003). Ganciclovir prophylaxis has been shown to inhibit allograft arteriosclerosis in CMV-infected rats (Lemstrom et al. 1994) as does the combination of ganciclovir and CMV hyperimmune globulin in seronegative recipients of a CMV seropositive heart transplant (Valantine et al. 2001). Statin-therapy also reduced the incidence of rejection episodes and the progression of cardiac allograft vasculopathy (Kobashigawa et al. 1995). Anti-hypertensive treatment with calcium channel blockers and ACE inhibitors had beneficial effects on allograft vasculopathy in the rat (Atkinson et al. 1993, Kobayashi et al. 1993, Paul et al. 1994). Similar results were achieved also in a small clinical study (Mehra et al. 1995).
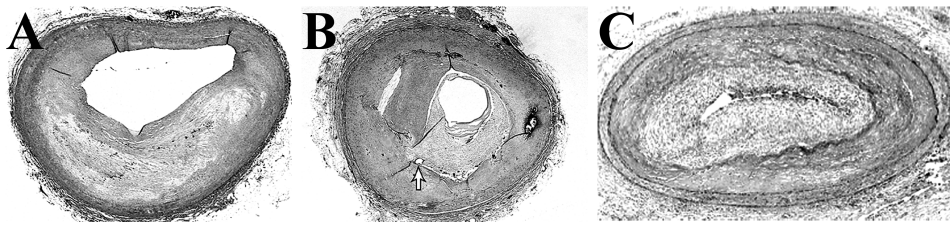
Figure 1. *Histological cross sections of a coronary artery with significant luminal obstruction caused by (A) atherosclerosis; (B) in-stent-restenosis after PTCA and stent implantation; and (C) cardiac allograft vasculopathy. The arrow indicates the hole where the stent wire has been removed. (A, B), Braunwald Medical Library, with permission of Current medicine, Philadelphia, PA; (C) Libby and Pober. Immunity 2001;14:387-397, with permission of Elsevier Inc., New York, NY.*

Finally, patient death with a functioning graft is the major cause of late graft failure after kidney transplantation, and accelerated atherosclerosis in the recipient accounts for about half of these deaths (Lindholm et al. 1995, Kasiske et al. 1996). Certain known risk factors of common atherosclerosis, such as age, sex, diabetes, smoking, and hypertension, are also associated with accelerated atherosclerosis in the recipient (Kasiske 1988). In addition, it seems that the inflammatory response evoked in the recipient may contribute to the development of atherosclerotic lesions. This is similar to several autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis, in which chronic inflammation and immune dysregulation are linked to accelerated coronary artery disease at young age (Manzi et al. 1997, Esdaile et al. 2001).

2. Pathogenesis of the vasculoproliferative disorders

2.1. The response-to-injury theory

Vascular response to injury is a common denominator in all the vasculoproliferative disorders described above. The response-to-injury theory, originally formulated and later modified by Russel Ross (Ross and Glomset 1973, Ross 1986, Ross 1993), suggests that the development of the lesion is a protective inflammatory-fibroproliferative response to different forms of injury to the arterial wall. Although originally established for atherosclerosis, it can also be applied to chronic rejection (Hayry et al. 1993, Libby and Pober 2001) and restenosis (Libby et al. 1992, Casscells et al. 1994), since the pathophysiological mechanisms following vascular injury are presumed not to be too distant.

2.2. Inflammation

Whether the vascular injury is mechanical, immunological, or infectious in origin, it initially leads to an inflammatory response. The injured endothelium is triggered to express numerous pro-inflammatory molecules, which mediate the entry of the inflammatory cells, mainly macrophages and lymphocytes, to the vessel wall. Selectins mediate the rolling of leukocytes along the endothelium by binding to carbohydrate ligands on the cells (Frenette and Wagner 1996, Frenette and Wagner 1997, Dong et al. 1998), and they are upregulated in endothelial cells overlying human atheromas (O'Brien et al. 1993), in restenosis (Serrano et al. 1997, Yasukawa et al. 1997), as well as in allograft arteriosclerosis (Koskinen and Lemstrom 1997). Further adhesion of leukocytes to the endothelium is mediated by vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Blocking the action of selectins, VCAM-1, and ICAM-1 has been shown to protect against the development of atherosclerosis, restenosis, or allograft arteriosclerosis (Sligh et al. 1993, Barron et al. 1997, Suzuki et al. 1997, Yasukawa et al. 1997, Collins et al. 2000).

Once leukocytes are adherent to the endothelium, they migrate into the vascular wall in response to the influence of different chemoattractant proteins, such as monocyte chemotactic protein-1 (MCP-1) (Gu et al. 1998). The activated inflammatory cells replicate and secrete cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), which, in turn, stimulate endothelial cells (EC) and vascular SMCs (Hancock et al. 1994). These cytokines are also produced by the ECs and SMCs (Moyer et al. 1991, Galea et al. 1996). Recent studies also support a role for interactions between CD40, expressed on lymphocytes, macrophages, ECs, and SMCs, and its ligand (CD40L) in the development of advanced atherosclerotic lesions, as the binding of CD40 to CD40L results in the production of cytokines, matrix-degrading proteases, and adhesion molecules (Schonbeck et al. 2000). Cytokine induction leads to increased expression of pro-inflammatory molecules, increased leukocyte extravasation, and increased inflammation - generating a vicious circle.

Finally, SMC migration and proliferation within the arterial intima is induced by growth factors and vasoactive peptides released by the activated leukocytes. SMCs express receptors for these ligands, and also synthesize them, which results in persistent paracrine and autocrine proliferation (Raines et al. 1989, Battegay et al. 1990) Finally, these inflammatory-

fibroproliferative cascades culminate in the advanced fibrous lesions in atherosclerosis, thickened neointima in restenosis, or vascular lesions in transplant arteriosclerosis.

2.3. Peptide growth factors

Peptide growth factors have an important role in the development of fibroproliferative vascular lesions (Cercek et al. 1991). Arterial SMC have been shown to synthesize numerous growth factors *in vitro* (Nilsson et al. 1985, Crowley et al. 1995). Several growth factors, such as insulin-like growth factor 1 (IGF-1) (Cercek et al. 1990), epidermal growth factor (EGF) (Miano et al. 1993), platelet-derived growth factor (PDGF) A and B (Majesky et al. 1990, Miano et al. 1993), and transforming growth factor β1 (TGF- β1) (Majesky et al. 1991) are upregulated in the vascular wall after endothelial injury *in vivo*. Furthermore, inhibition of PDGF (Ferns et al. 1991b, Myllarniemi et al. 1997, Sihvola et al. 1999, Noiseux et al. 2000), IGF (Hayry et al. 1995), and basic fibroblast growth factor (bFGF) (Lindner and Reidy 1991) signalling with antibodies, peptidomimetics, antisense treatment, or tyrosine kinase inhibitors have been shown to prevent injury-induced neointimal hyperplasia and allograft arteriosclerosis - further emphasizing their role in these disease processes. In this thesis, I have focused mainly on the regulation of PDGF and IGF-1 in the vascular response-to-injury.

2.4. Platelet-derived growth factor (PDGF)

PDGF is known as the most potent SMC chemoattractant and mitogen. It is also important in embryonic vascular development (Leveen et al. 1994, Soriano 1994) and has angiogenic properties (Battegay et al. 1994). Although originally discovered as a product of platelets (Antoniades et al. 1979), PDGF has thereafter been shown to originate from several other cell types, including vascular ECs and SMCs (DiCorleto and Bowen-Pope 1983, Nilsson et al. 1985). Four PDGF genes have been identified; the classical PDGF-A and PDGF-B, and the novel PDGF-C and PDGF-D. PDGF ligands consist of disulfide-linked polypeptide dimers. PDGF-A and PDGF-B form both homo- and heterodimers (PDGF-AA, -BB, or -AB) (Ross et al. 1986), while the novel PDGFs exist only as homodimers (PDGF-CC and PDGF-DD) (Li et al. 2000, Bergsten et al. 2001). PDGF ligands act via PDGF-receptors (PDGF-Rα and -Rβ) (Heldin and Westermark 1999). PDGF-Rα binds PDGF-A, PDGF-B, and PDGF-C, whereas PDGF-Rβ binds only PDGF-B and PDGF-D (Hart et al. 1988, Claesson-Welsh et al. 1989, Li et al. 2000, Bergsten et al. 2001). PDGF-receptors are protein tyrosine kinase receptors. Upon

ligand binding, the receptors dimerize, which results in autophosphorylation of the tyrosine residues between the two receptors and a biological response.

PDGF and its receptors are detected in normal human arteries (Barrett and Benditt 1988) and they are upregulated in human atherosclerotic arteries (Barrett and Benditt 1988, Libby et al. 1988, Wilcox et al. 1988), coronary arteries after PTCA (Tanizawa et al. 1996, Ueda et al. 1996), and transplant vasculopathy (Zhao et al. 1995), as well as in several experimental models of vascular injury (Majesky et al. 1990, Uchida et al. 1996, Lemstrom and Koskinen 1997). *In vitro,* PDGF-A mainly acts as a mitogen for vascular cells, and PDGF-Rα, when activated by ligand binding, stimulates proliferation, but not migration, of vascular SMC (Koyama et al. 1994). PDGF-B, on the other hand, is considered as the most potent SMC mitogen and chemoattractant (Grotendorst et al. 1982, Ross et al. 1986). Infusion of PDGF-BB into rats after denudation injury (Jawien et al. 1992) or transfer of the recombinant PDGF-B gene into porcine arteries (Nabel et al. 1993) induced intimal hyperplasia *in vivo*. On the contrary, neointimal thickening was inhibited by a neutralizing PDGF antibody (Ferns et al. 1991b) as well as by antisense treatment against PDGF-Rβ (Noiseux et al. 2000).

The roles of PDGF-C and PDGF-D in the vasculature are being explored. So far, both ligands have been demonstrated to stimulate human coronary artery SMC proliferation *in vitro* (Uutela et al. 2001).

2.5. Insulin-like growth factor-1 (IGF-1)

IGF-1 has numerous physiological actions from embryonic development (Baker et al. 1993) to metabolic functions (Jacob et al. 1989), and as a mediator of the effects of growth hormone. It is also an important regulator of proliferation and migration of vascular cells, including fibroblasts and SMC (Cercek et al. 1990, Ferns et al. 1991a, Bornfeldt et al. 1994). The actions of IGF-1 are mediated via a specific membrane tyrosine kinase receptor, IGF-1R (LeRoith et al. 1995), which has been detected on SMC (Pfeifle and Ditschuneit 1983), inflammatory cells (Hochberg et al. 1992), and ECs (Bar and Boes 1984). The bioactivity of IGF-1 is, furthermore, regulated by six different IGF binding proteins (IGFBP) (Shimasaki and Ling 1991).

IGF-1 is expressed in normal SMCs of rat aorta, localizing predominantly to medial SMC (Delafontaine et al. 1991, Khorsandi et al. 1992), and it is upregulated markedly after vascular injury in rat (Cercek et al. 1990) and in human coronary atherectomy specimens (Grant et al. 1996). IGF-1 is also synthesized by macrophages (Nagaoka et al. 1990) and ECs (Delafontaine 1995). IGF-1 alone appears to be a poor mitogen for vascular SMC (Ferns et al. 1991a). However, IGF-1 is considered as an important progression factor for PDGF, in other words, autocrine or paracrine production of IGF-1 and a functional IGF-1R are suggested to be necessary for PDGF to induce its full mitogenic effects on SMCs and fibroblasts (Clemmons and Van Wyk 1985, Sara and Hall 1990, Miura et al. 1994, DeAngelis et al. 1995).

2.6. Vascular precursor cells

Traditionally, vascular SMCs were believed to exist in two different phenotypic states, the contractile and the secretory state (Campbell and Campbell 1990, Thyberg et al. 1990). SMCs within a normal artery were described as contractile, as they were filled with myofilaments but contained a poorly developed Golgi apparatus and rough endoplasmic reticulum. In response to the action of different cytokines and growth factors in the injured artery, these 'normal' SMC were thought to undergo a phenotypic change into a secretory or synthetic phenotype. This phenotype had histologically an abundant rough endoplasmic reticulum and Golgi apparatus, but few or no myofilaments. The secretory SMCs were capable of producing a number of ECM proteins and matrix metalloproteinases, that enabled their migration into the intima (Bendeck et al. 1994, Galis et al. 1995, Bendeck et al. 1996), as well as growth factors.

Until recently, it was also considered that the SMCs accumulating in the intima migrated there only from the medial layer. Recent data, however, suggest that the proliferating neointimal SMC in transplant vasculopathy, in experimental atherosclerosis, and after mechanical arterial injury derive mainly from circulating somatic stem cells (vascular progenitors), partly of bone marrow origin, that at least *in vitro* are capable of differentiating into endothelial and smooth muscle cells (Hillebrands et al. 2000, Hillebrands et al. 2001, Saiura et al. 2001, Shimizu et al. 2001, Hillebrands et al. 2002, Hu et al. 2002, Sata et al. 2002). The route of vascular progenitors to the vessel wall is not clear. Some studies suggest that adventitial activation contributes significantly to neointima formation (Shi et al. 1996a, Shi et al. 1996b, Frosen et al. 2001) while others support a luminal route to the vascular wall (Matsumoto et al. 2003).

3. Estrogens

3.1. Introduction to natural estrogens

Estrogens are steroid hormones that regulate the growth, differentiation, and function of several tissues in the human body. The female reproductive system, including mammary gland, uterus, placenta, and vagina, is considered as a traditional estrogen target tissue. In addition, estrogens play an important role in the male reproductive system and many non-reproductive tissues, such as the cardiovascular system, bone, and the central nervous system.

In premenopausal women, 95% of the estrogens are synthesized in the granulosa cells of the ovary (Richards and Hedin 1988). The most important natural estrogen in premenopausal women is 17β-estradiol (E2) (Fig. 2). The level of E2 production depends on the time of the menstrual cycle, and serum concentrations of E2 are approximately 40-360 pmol/l in the follicular phase, 630-2800 in the midcycle, and 700-1200 pmol/l in the luteal phase of the menstrual cycle, and up to 70,000 pmol/L during pregnancy (O'Malley and Strott 1999). In the plasma, E2 is bound predominantly to albumin and sex hormone-binding globulin (SHBG) (Dunn et al. 1981) and the remaining free fraction is available for uptake by estrogen target tissues. Estradiol is mainly excreted in the urine, and the principal metabolites are estriol (E3) and catechol estrogens (2- and 4-hydroxy-estrone), which also have numerous biological actions (Dubey et al. 2004).

During the menopause, the ovaries cease to secrete E2, and thereafter, estrogens are mainly derived from androgens, such as testosterone, androstenedione, and dehydroepiandrosterone, through peripheral aromatization in mesenchymal cells of the adipose tissue, the osteoblasts and chondrocytes of bone, the vascular endothelium and SMCs as well as the brain (Grodin et al. 1973, Simpson 2003). In postmenopausal women estrone (E1), which has a biologic potency of approximately one third that of estradiol, is the predominant estrogen. In males, estrogens are produced by the testes, adrenals, and adipose tissue, and the levels of E2 are similar to those of postmenopausal women (30-200 pmol/L) (O'Malley and Strott 1999).
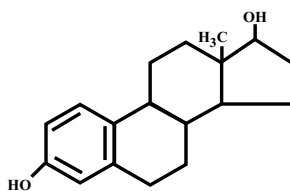
Figure 2. *Molecular structure of 17β-estradiol*

3.2. The nuclear receptor superfamily

Most of the effects of estrogen are mediated by estrogen receptors (ER). ERs belong to the nuclear receptor (NR) gene superfamily, which also includes receptors for other steroid hormones, thyroid hormones, vitamin A and D, retinoids, as well as several 'orphan receptors', which are receptors interacting with unknown compounds (Evans 1988, Laudet et al. 1992, Mangelsdorf et al. 1995).

The NR superfamily has an evolutionarily and functionally conserved structure (Kumar et al. 1987, Laudet et al. 1992). The receptors consist of six functional domains: an N-terminal domain (A/B domain), a DNA-binding domain (DBD; C domain), a hinge region (D domain), and a ligand-binding domain (LBD; E/F domain). The A/B domain, which usually has the lowest degree of sequence similarity among the NR family members, contains a ligand-independent transactivation function (AF-1) domain that contributes to transcriptional activity interacting with other transcription factors and coregulatory proteins, and it acts in a promoter- and cell-specific fashion (Tora et al. 1989, Kraus et al. 1995, McInerney and Katzenellenbogen 1996, McInerney et al. 1996). The DBD is the most conserved region among the NR superfamily. It contains two zinc finger motifs, and it is involved in DNA binding, receptor dimerization, and transactivation of genes (Freedman 1992, Glass 1994). The D domain serves as a hinge between DBD and LBD, and its actions are less well characterized. LBD is critical in the binding of NR agonists and antagonists as well as dimerization, cofactor binding, ligand-dependent transactivation, and nuclear localization, and it is relatively well conserved among nuclear receptors (Kumar et al. 1987, Carson-Jurica et al. 1990). A second activation function (AF-2) domain is located in the LBD and it interacts with coregulatory proteins (Kumar et al. 1987, Webster et al. 1988, Tora et al. 1989).

3.3. Nuclear receptor activation and signal transduction

Nuclear receptors act as ligand-activated transcription factors. In short, their activation involves diffusion of the ligand to the target cell (Rao 1981) and binding of the ligand to the receptor in the cytoplasm or the nucleus. Ligand binding detaches the receptor from inhibitory heat shock proteins (hsp) and induces a conformational change in the structure of the receptor, which facilitates receptor dimerization, nuclear translocation, and the binding of the ligand-receptor complex to specific DNA elements in the target genes (Tsai and O'Malley 1994). This results in the regulation of the gene transcription through interaction with coregulatory proteins (coactivators or corepressors) and the transcription machinery (Fig. 3). Receptor activation is also influenced by phosphorylation (Kuiper and Brinkmann 1994).

Furthermore, nuclear receptors have been shown to regulate the transcription of some genes that do not contain their classical response elements. For example, estrogen stimulates several genes through interactions with other transcription factors, such as AP-1 or Sp1, that bind to their cognate DNA binding sites (Webb et al. 1995, Saville et al. 2000). Estradiol can also inhibit the expression of target genes through negative regulation of transcription factors, including nuclear factor-κB (Ray et al. 1994, Galien and Garcia 1997).

On the other hand, nuclear receptors can also be activated in a ligand-independent fashion, by signals originating from the cell surface. Estrogen receptors are activated in the absence of ligand by activators of protein kinase A, protein kinase C, dopamine, and growth factors, such as EGF and IGF-1 (Power et al. 1991, Aronica and Katzenellenbogen 1993, Smith et al. 1993, Kato et al. 1995, Bunone et al. 1996, Weigel 1996, Lahooti et al. 1998).
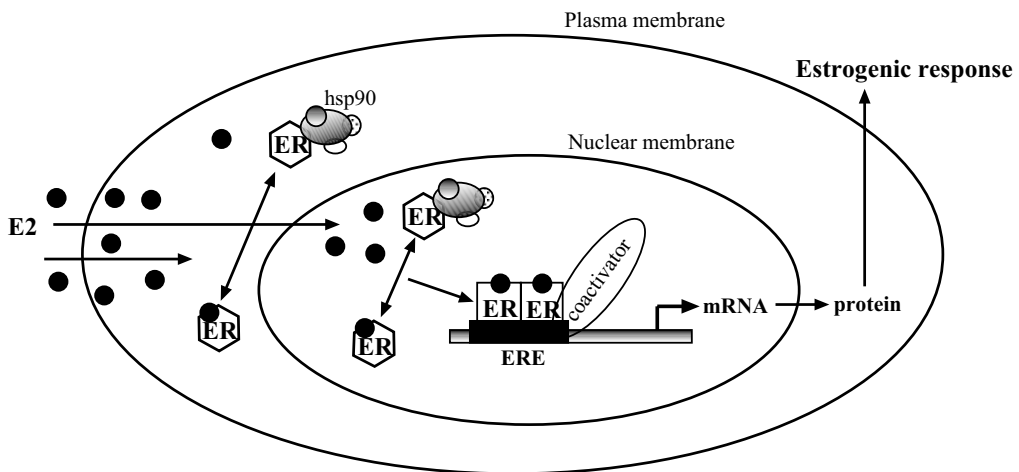
Figure 3. *The classical pathway of estrogen receptor action in the cell. Estradiol (E2) diffuses into the cell and binds to the estrogen receptor (ER) in the cytoplasm or the nucleus. ER undergoes a conformational change and dissociates from heat shock proteins (hsp), such as hsp90. The ligand-receptor complex dimerizes and interacts with specific DNA elements in the nucleus, such as estrogen response element (ERE), located in the promotor region of the target genes, as well as with coregulatory proteins. This results in the up- or downregulation of the gene transcription and an estrogenic response in the cell.*

3.4. Estrogen receptors

The existence of the estrogen receptor was recognized about 40 years ago by the observation that uterus and vagina specifically bound estradiol (Jensen and Jacobson 1962). The receptor was cloned 25 years later (Green et al. 1986, Greene et al. 1986), and thereafter, the ER was believed to be the only mediator of estrogen's action in its target tissues. Surprisingly, however, ER knock-out mice (ERKO) appeared healthy, except for problems in their fertility (Lubahn et al. 1993, Korach et al. 1996), and furthermore, mutation of the estrogen receptor gene in man was not lethal (Smith et al. 1994). Then, in 1996 a novel ER was cloned in the rat (Kuiper et al. 1996), man (Mosselman et al. 1996), and mouse (Tremblay et al. 1997), and it was named ERβ to distinguish it from the classical receptor, which was renamed ERα. In many respects, the finding of this new ER opened fresh vistas for the understanding of estrogen receptor signalling.

Both ERα and ERβ bind E2 with high affinity (Kuiper et al. 1997) and they bind to the classical estrogen response elements (ERE) with similar affinities (Kuiper et al. 1996, Kuiper et al. 1997). They share about 96% structural homology in their DBD and 53% homology in their LBD (Kuiper et al. 1996), which suggests that the receptors bind similar DNA elements

but may have a distinct spectrum of ligands. Indeed, the receptors differ in their affinities for several natural and synthetic compounds (Barkhem et al. 1998, Kuiper et al. 1998). The ERβ protein is smaller than that of ERα and the receptors differ markedly in their N-terminal AF-1 domain and the C-terminal AF-2 domain (Fig. 4). This suggests that the transcriptional activation of different estrogen-responsive genes may be different due to promoter and cell-specific factors and by different interactions of the N- and C-terminal domains with proteins in the transcription complexes; for example, ERα and ERβ have opposite functions at the AP-1 site (Paech et al. 1997). The human ERβ gene shows approximately 89% identity to that of rat, 88% to mouse, and 47% to human ERα (Enmark et al. 1997).

Several splicing variants for both ERs have been identified (Fuqua et al. 1993, Friend et al. 1995, Zhang et al. 1996, Chu and Fuller 1997, Leygue et al. 1998, Moore et al. 1998, Petersen et al. 1998, Hanstein et al. 1999, Hodges et al. 1999, Li et al. 2003). The biological and physiological roles of most ERα splice variants have not been clarified. Some ERβ splice variants, such as ERβ-503 and ERβcx, do not bind E2 and they have also been reported to have a dominant negative effect on ERα and ERβ-mediated activation of target genes (Maruyama et al. 1998, Ogawa et al. 1998). ERβcx has been of particular interest due to its expression in human cancers (Ogawa et al. 1998).
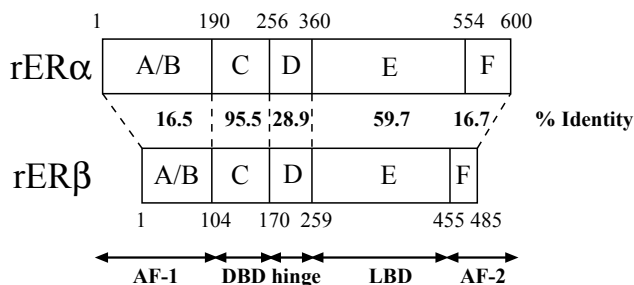
Figure 4. *Comparison between rat ERα and ERβ protein. The bars represent rat ER molecules divided into their structural domains. Amino acid positions are indicated at region boundaries. The percentage of amino acid identity in the A/B (AF-1, N-terminal), C (DNA binding), D (hinge), E (ligand binding), and F (AF-2, C-terminal) domains is demonstrated. Similar homologies are present in the respective domains of human and mouse ER subtypes. Modified from Kuiper and Gustafsson FEBS Lett. 1997;410:87-90, with permission of Elsevier Inc., New York, NY.*

3.5. Estrogen receptor expression

Estrogen receptors have been demonstrated in numerous rat, mouse, and human tissues both at the mRNA (Kuiper et al. 1996, Mosselman et al. 1996, Couse et al. 1997, Enmark et al. 1997, Kuiper et al. 1997, Tremblay et al. 1997) and at the protein (Saunders et al. 1997, Taylor and Al-Azzawi 2000) level. The receptors have clearly different tissue distribution, and when co-expressed in the same tissue, they usually have a distinct cell-specific localization. ERα is predominantly expressed in the uterus, placenta, testis, kidney, and liver. ERβ, on the other hand, predominates in many non-classical target tissues including the ovary, prostate epithelium, bladder, lung, the gastrointestinal (GI) tract, spleen, thymus, lymph nodes, and bone marrow. Both receptors are found in the kidney, mammary gland, different regions of brain, bone, adrenal gland, the cardiovascular system, and epididymis. There may, however, be some species-specific variance in the expression pattern (Kuiper et al. 1996, Enmark et al. 1997).

The co-expression of ERα and ERβ in some tissues suggests that the receptors may interact with each other. It has been demonstrated that, in addition to binding DNA as homodimers, the ERs also form heterodimers, which may increase their functional capability to regulate gene expression (Cowley et al. 1997, Pettersson et al. 1997). For example, although the uterus predominantly expresses ERα, it is not free of ERβ, and it appears that ERβ modulates the uterotrophic responses of ERα in the uterus (Weihua et al. 2000, Lecce et al. 2001).

In the vasculature, estrogen receptors have been detected in the arteries and veins of several species (Malinow et al. 1963, McGill and Sheridan 1981, Horwitz and Horwitz 1982, Lin and Shain 1985, Perrot-Applanat et al. 1988, Orimo et al. 1993). They are present in vascular endothelial (Colburn and Buonassisi 1978, Kim-Schulze et al. 1996, Venkov et al. 1996) and smooth muscle cells (Nakao et al. 1981, Karas et al. 1994, Losordo et al. 1994, Bayard et al. 1995, Register and Adams 1998, Hodges et al. 2000, Taylor and Al-Azzawi 2000) as well as in cardiac myocytes (Grohe et al. 1997, Saunders et al. 1997, Grohe et al. 1998) and fibroblasts (Grohe et al. 1997). Vascular cells also express variant forms of the ERs (Karas et al. 1995, Inoue et al. 1996, Chu and Fuller 1997, Hodges et al. 1999, Li et al. 2003).

In addition to the classical nuclear receptors, plasma membrane estradiol binding sites have been identified in vascular and other tissues (Razandi et al. 1999), and these mediate rapid

intracellular signalling cascades (Chambliss et al. 2000, Russell et al. 2000, Chambliss et al. 2002, Li et al. 2003). Finally, changes in plasma estrogen concentration regulate the levels of ERs in the tissues. ER levels in the cytosol of human artery were highest in the late follicular phase of the menstrual cycle (Lantta et al. 1983, Leiberman et al. 1990). Moreover, estrogen treatment caused a redistribution of the ER from the cytoplasmic to nuclear compartment in the rabbit aorta (Lin and Shain 1985).

3.6. The role of gender and estrogens in different vasculoproliferative disorders

Coronary heart disease is a major cause of morbidity and mortality in both men and women. The incidence of CHD and the related cardiovascular complications is rare in premenopausal women (Barrett-Connor and Bush 1991, Isles et al. 1992). However, the risk increases markedly after the menopause (Kannel et al. 1976, Colditz et al. 1987, van der Schouw et al. 1996, Jacobsen et al. 1997, Joakimsen et al. 2000), and compared to men, there is a 5- to 10-year delay in the onset of the disease in women (Wenger 1997, Maxwell 1998) (Fig. 5).
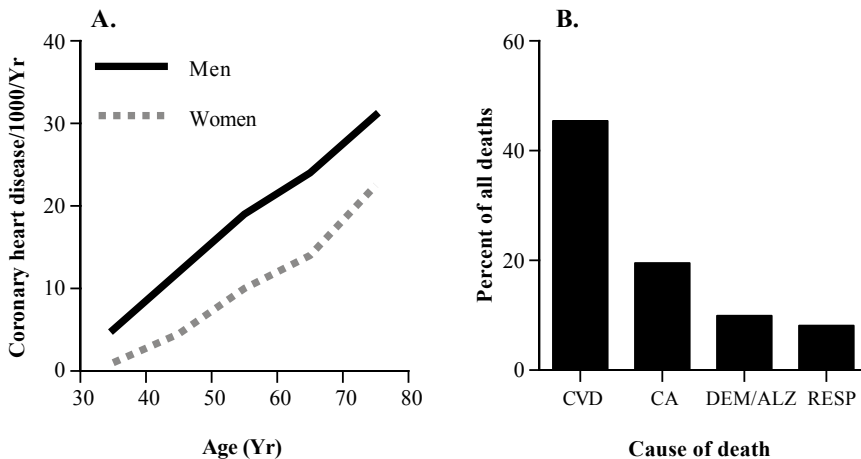
Figure 5. *A. Annual rate of coronary heart disease in men (solid line) and women (dashed line) from the Framinghman Heart Study. Modified from Castelli WP. Am. J. Obstet. Gynecol. 1988;158(6 Pt 2):1553-60, with permission of Elsevier Inc., New York, NY. **B**. Leading causes of death of women in Finland in 1999. Finnish Heart Association, Helsinki, Finland, with permission. CVD, cardiovascular disease; CA, cancer; DEM, dementia; ALZ, Alzheimer's disease; RESP, respiratory disease.*

These observations are most likely due to the protective effect of endogenous female sex hormones, and particularly of estrogens. This hypothesis has been supported by population studies, which suggest that in primary prevention, women using postmenopausal hormone replacement therapy (HRT) have approximately a 35% to 50% lower rate of CHD (Stampfer et al. 1985, Stampfer et al. 1991, Grady et al. 1992, Grodstein et al. 1996, Grodstein et al. 2000). However, in the secondary prevention of cardiovascular disease, HRT does not seem to offer any overall benefit (Hulley et al. 1998, Herrington et al. 2000).

Male gender has been associated with increased risk for post-angioplasty restenosis (Holmes et al. 1984). On the other hand, the short-term results of PTCA in women have been considered less satisfactory with lower initial success rate and higher mortality rate than in men. Many of the differences may be explained by worse baseline characteristics in women, such as older age, unstable angina, congestive heart failure, diabetes, hypertension, and hypercholesterolemia, as well as smaller vessel diameter, and more coronary tortuosity. The long-term results after PTCA are comparable or better in women, with similar symptomatic improvement, lower rates of restenosis, and improved survival compared to men (Cowley et al. 1985, Bell et al. 1993, Kelsey et al. 1993, Arnold et al. 1994, Weintraub et al. 1994, Bell et al. 1995, Ruygrok et al. 1996). Also with coronary stents, long-term survival is better or equal in women than in men (Alfonso et al. 2000, Antoniucci et al. 2001, Mehilli et al. 2003). There are few data on the effects of HRT on the outcome after PTCA and the results are inconsistent (O'Keefe et al. 1997, Abu-Halawa et al. 1998, Khan et al. 2000, Khan et al. 2003). Recently, estrogen-releasing stents have been introduced, and preliminary results are promising (New et al. 2002, Joung et al. 2003, Abizaid et al. 2004)

Gender also influences the outcome in experimental and clinical transplantation. In general, male recipients of a female transplant are at increased risk for rejection, allograft vasculopathy, and graft loss (Keogh et al. 1991, Mehra et al. 1994, Marino et al. 1995, Brooks et al. 1996, Vereerstraeten et al. 1999, Zeier et al. 2002) (Fig. 6). Allografts from male donor have a better 1-year survival rate, since acute rejection episodes are more common in females (Cecka 1986, Esmore et al. 1991, Fabbri et al. 1992, Neugarten and Silbiger 1994, Brooks et al. 1996, Meier-Kriesche et al. 2001). However, evidence exists that long-term allograft outcome is improved in female recipients (Neugarten and Silbiger 1994, Meier-Kriesche et al. 2001).
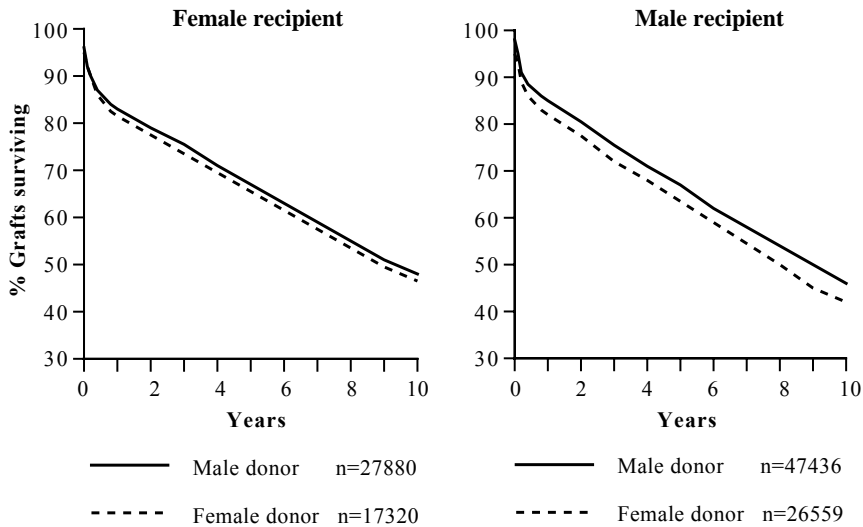
Figure 6. *Donor gender-related differences in the survival of first cadaveric kidney transplants performed between 1985 and 2000. Kidney transplants from female donors had a significantly lower survival rate, particularly in male recipients. Zeier et al. J Am Soc Nephrol 2002;13:2570-2576, with permission of the authors and Lippincott Williams & Wilkins, Baltimore, MD.*

Reasons for the gender-related differences are largely unknown. Some studies suggest that nephron underdosing, i.e. a smaller kidney size in females versus males, may play a role (Brenner et al. 1992, Mackenzie et al. 1995). Antigenicity and immunological factors are probably also important. HLA mismatching reduces the survival of female donor grafts more than that of male grafts (Cecka 1986). Broadly sensitized recipients have a lower 1-year survival rate when they receive a female kidney (Zhou and Cecka 1989). Females, especially those who have been pregnant, are also more frequently sensitized than males (Suciu-Foca et al. 1983, Koka and Cecka 1989, Regan et al. 1991, van Kampen et al. 2001). However, gender-related differences in graft survival are seen even in HLA-identical siblings, possibly pointing to an additional role of non-HLA factors (Zeier et al. 2002). An alternative view is that, sex hormones, not the sex of the donor, are responsible for the recipient gender-related differences. In ovariectomized female rats, progression of chronic allograft nephropathy is more rapid in allografts of female donor than of male donor (Muller et al. 1999). However, estrogen supplementation prevents the functional decline of female donor grafts whereas testosterone increases rejection in kidneys of male origin (Muller et al. 1999). Moreover, testosterone impairs long-term allograft outcome in male animals, while estrogen improves allograft survival in female animals, irrespective of the donor gender (Antus et al. 2002).

The role of estrogen in vasculoprotection has been intensely studied in experimental models. Endogenous estrogens protect female rats against neointimal thickening and gonadectomy abolishes the effect (Chen et al. 1996). Estrogen treatment inhibits fatty streak and lesion formation in ApoE deficient mice (Bourassa et al. 1996, Elhage et al. 1997). Moreover, it inhibits neointima formation after carotid balloon injury in rabbits (Foegh et al. 1994), rats (Chen et al. 1996), and mice (Sullivan et al. 1995); immunologically-induced vascular fibroproliferative dysplasia in rabbit aorta (Cheng et al. 1991) and cardiac allografts (Foegh et al. 1987); as well as diet-induced coronary artery atherosclerosis in monkeys (Adams et al. 1987, Adams et al. 1990).

3.7. Mechanisms behind estrogen's vasculoprotective effect

Despite years of intense study, the mechanisms behind estrogen's vasculoprotective effects are still incompletely understood. Estrogens have beneficial effects on serum lipid concentrations (Stevenson et al. 1993, The Writing Group for the PEPI Trial 1995); however, evidence exists that estrogen-induced alterations in the lipids can account for only approximately one third of the observed vasculoprotective effects (Bush et al. 1987, Grady et al. 1992, Mendelsohn and Karas 1999). Thus, the direct estrogenic effects on the vascular wall most probably make up a major part of its vasculoprotective properties. Estrogen is believed to exert its effects both directly via a rapid non-genomic pathway, such as the rapid vasodilatory effect (Farhat et al. 1996a) (Table 2), and via a genomic pathway through nuclear ERs, which regulate the transcription of target genes (Table 3). The non-genomic effects occur within minutes while the genomic effects take place over hours.

Table 2. Potential non-genomic mechanisms behind estrogen's vasculoprotective actions

Physiologic or pathophysiologic effect of E2	Potential mechanism	References
Vasodilation	Changes in membrane ionic permeability, particularly Ca^{2+} and K^+ ion channel functions	Farhat et al. 1996a, Mendelsohn and Karas 1999, Dubey and Jackson 2001
	Upregulation of cAMP and cGMP	Farhat et al. 1996a, Dubey and Jackson 2001
	Increased production and release of NO from ECs, possibly through activation of eNOS by ERα located in the plasma membrane	Mendelsohn and Karas 1999, Dubey and Jackson 2001

The development of ERα, ERβ, and ER(α,β) double knock out-mice has increased our understanding of the ER-mediated effects in the vasculature. Vascular SMCs isolated from ERβ-deficient mice have abnormalities in ion channel function, and they develop sustained systolic and diastolic hypertension with age (Zhu et al. 2002). Estrogen's vasculoprotective effects on the vascular injury response were preserved in mice lacking either ERα or ERβ, but not in mice lacking both ERs (Iafrati et al. 1997, Karas et al. 1999, Karas et al. 2001), which suggested that they are mediated by the ERs, and that either of the known ERs is sufficient to protect against vascular injury. However, the mice developed no neointima, and the analysis was based only on changes in the medial layer. This differs markedly from the case in other animals and man, which makes it difficult to evaluate the relative roles of the two receptors in vasculoprotection.

Table 3. Potential genomic mechanisms behind estrogen's vasculoprotective actions

Physiologic or pathophysiologic effect of E2	Potential mechanism	References
Vasodilation	Upregulation of prostacyclin synthase and eNOS	Farhat et al. 1996b, Mendelsohn and Karas 1999, Dubey and Jackson 2001
	Downregulation of renin, ACE, ET-1, and AT 1 receptor	
Cell adhesion	Downregulation of VCAM, ICAM, E-, and P-selectin	Caulin-Glaser et al. 1998, Cushman et al. 1999, Mendelsohn and Karas 1999, Zanger et al. 2000, Dubey and Jackson 2001, Oger et al. 2001
Endothelial recovery (reendothelialization)	Upregulation of bone marrow-derived endothelial progenitor cell production	Farhat et al. 1996b, Mendelsohn and Karas 1999, Dubey and Jackson 2001, Iwakura et al. 2003, Strehlow et al. 2003b
	Stimulation of EC replication, possibly via upregulation of VEGF and bFGF	
	Inhibition of EC apoptosis	
SMC function	Inhibition of MAP kinase activity and the subsequent SMC proliferation and migration	Suzuki et al. 1996, Dubey and Jackson 2001, Takahashi et al. 2003
	Downregulation of IGF-1, IGF-1R, c-fos, c-myc, and cell cycle proteins	
Extracellular matrix synthesis	Downregulation of collagen and elastin synthesis	Farhat et al. 1996b, Mendelsohn and Karas 1999, Zanger et al. 2000, Dubey and Jackson 2001
	Upregulation of matrix metalloproteinases	
Lipid metabolism LDL↓, HDL↑ triglycerides↑	Upregulation of apolipoproteins A and E, LDL receptor, and lipoprotein lipase	Mendelsohn and Karas 1999, Dubey and Jackson 2001
	Downregulation of apolipoprotein B and lipoprotein(a)	
Inflammation	Downregulation of IL-1, IL-6, MCP-1, MHC II, and TNF-α	Saito et al. 1997, Mendelsohn and Karas 1999, Srivastava et al. 1999, Dubey and Jackson 2001, Koh et al. 2001
Coagulation, thrombosis, and fibrinolysis	Downregulation of fibrinogen, antithrombin III, protein S, PAI-1	Mendelsohn and Karas 1999, Dubey and Jackson 2001
	Upregulation of TPA	
Antioxidant effect	Downregulation of free radical production, inhibition of LDL oxidation	Dubey and Jackson 2001, Laufs et al. 2003, Strehlow et al. 2003a
	Upregulation of superoxide dismutase	

3.8. Estrogen receptors as targets for vasculoprotective drug therapies

Although the vasculoprotective properties of E2 have been widely demonstrated both in epidemiological and experimental studies, its effects have been difficult to understand and exploit due to harmful side-effects in the reproductive system: while being antiproliferative in the vasculature, estrogen-treatment increases the risk for endometrial and breast cancer (Key and Pike 1988, Barrett-Connor 1992, Colditz et al. 1995).

However, the discovery of ERβ and the distinct tissue distribution and transcriptional regulation of the two ERs prompted us to generate a hypothesis that perhaps estrogen's different effects in different tissues could be separated with receptor subtype-selective, i.e. tissue-selective compounds - and the hypothesis was subsequently proven to be correct (Makela et al. 1999). An ideal compound should have estrogenic effects in the cardiovascular system and bone, while being neutral in breast and uterus.

3.9. Selective estrogen receptor modulators (SERM)

SERMs are compounds that act as ER agonists in some tissues while being antagonists in others (MacGregor and Jordan 1998, Morello et al. 2002). SERMs act on their target cells by diffusing into the cell and binding to the two ERs with variable affinities. According to current knowledge, the action of a SERM in a given tissue is dependable on several factors, such as the ER subtype-selectivity of the compound, the predominant ER expressed in the tissue, and the nature of the target gene promoter (Webb et al. 1995, Paech et al. 1997, Jones et al. 1999). The conformational change that takes place upon binding of the SERM to either of the ERs, and particularly, the position of helix 12 in the LBD, depends on the SERM involved, and it plays a critical role in the agonist or antagonist configuration of the ER that determines the ability of the ER-ligand complex to interact with coregulatory proteins and exert either the estrogen agonist or antagonist response in the tissue (Martin et al. 1988, McDonnell et al. 1995, Brzozowski et al. 1997, Shiau et al. 1998, Paige et al. 1999, Pike et al. 1999). Also, the expression of coregulatory proteins has been shown to vary in different cells and tissues (Voegel et al. 1996, Kalkhoven et al. 1998, Kurebayashi et al. 2000)

The SERMs currently in clinical use, namely tamoxifen, raloxifene, and toremifene, are used for the prevention and treatment of breast cancer and postmenopausal osteoporosis (Fig. 7). In

addition, several promising compounds have been described, including idoxifene and ospemifene (Pace et al. 1997, Nuttall et al. 1998, DeGregorio et al. 2000, Qu et al. 2000, Taras et al. 2001) (Fig. 7). In general, SERMs have minimal effects on uterine endometrium. Tamoxifen, however, is associated with a 2-6 fold increased risk for endometrial cancer, which has limited its use outside chemotheraphy (Bernstein et al. 1999, Bergman et al. 2000).

The data presently available on the role of SERMs in vasculoprotection are promising. Tamoxifen and raloxifene have been reported to have beneficial effects on lipids (Walsh et al. 1998, Wenger and Grady 1999, De Leo et al. 2001), to reduce markers of cardiovascular risk (Love et al. 1994, Walsh et al. 1998), and to be associated with lower rates of cardiovascular events in humans (McDonald et al. 1995, Barrett-Connor et al. 2002). Both are also favourable in lipid-induced experimental atherosclerosis (Bjarnason et al. 1997, Reckless et al. 1997, Williams et al. 1997), although contradictory results also exist (Clarkson et al. 1998, Castelo-Branco et al. 2004). Although ospemifene has a cholesterol-lowering effect in the rat (Qu et al. 2000), it showed a neutral effect on vascular markers in a recent clinical study (Ylikorkala et al. 2003). Only some data are available on the effects of SERMs on restenosis: raloxifene and idoxifene inhibited neointima formation in the rat (Kauffman et al. 2000, Yue et al. 2000).
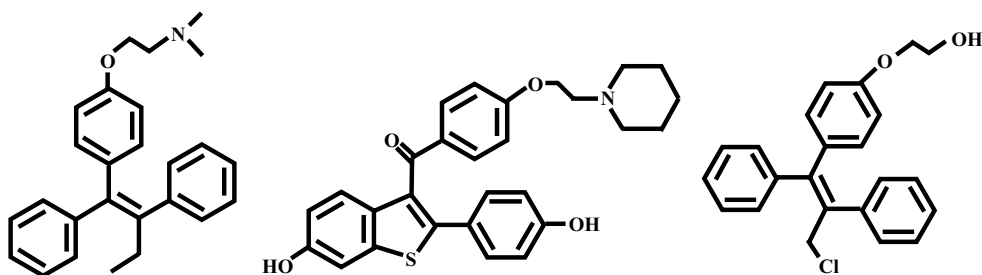
Figure 7. *Molecular structures of tamoxifen (left), raloxifene (middle), and ospemifene (right).*

3.10. Phytoestrogens

Phytoestrogens are a large family of plant-derived molecules possessing various degrees of estrogenic activity. They are present in many food products, such as soy and rye, as well as in many food supplements. Phytoestrogens are traditionally divided into three main groups, namely isoflavones, lignans, and cumestans, of which isoflavones comprise the most common group (Albertazzi and Purdie 2002). The two major isoflavones are genistein and daizein, and

their major natural sources are soy and red clover (Eldridge and Kwolek 1983). Epidemiological studies suggest that phytoestrogen-rich diet is associated with reduced incidence of breast and prostate cancer, cardiovascular disease, osteoporosis, and climacteric symptoms (Setchell et al. 1984, Adlercreutz et al. 1991, Somekawa et al. 2001).

Phytoestrogens' cardiovascular benefits have been shown in several studies, where they have improved plasma lipid levels (Anderson et al. 1995, Anthony et al. 1996, Baum et al. 1998), vascular reactivity, and arterial compliance (Honore et al. 1997, Nestel et al. 1997, Figtree et al. 2000), and had antioxidant effects (Tikkanen et al. 1998, Deodato et al. 1999, Jenkins et al. 2000), as well as reduced experimental atherosclerosis (Yamakoshi et al. 2000, Alexandersen et al. 2001, Clarkson et al. 2001).

Phytoestrogens bind to the ERs, and as with SERMs, the conformational change of the receptor, and especially the position of helix 12 of the LBD, differs depending on the type of ligand that binds to the receptor (Pike et al. 1999). Thus, these compounds could also be called 'phytoSERMs'. Genistein (Fig. 8) has approximately 20 times higher binding affinity for ERβ than ERα (Kuiper et al. 1997). In micromolar concentrations, genistein also inhibits several enzymes, such as tyrosine kinase, which may contribute to its effects, particularly its antiproliferative actions, in high doses (Akiyama et al. 1987).
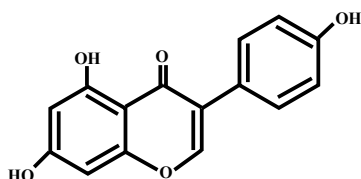
Figure 8. *Molecular structure of genistein.*

AIMS OF THE STUDY

This study was based on the observation that there are two estrogen receptors that differ in their tissue distribution and regulation of gene transcription, which might explain why estrogen can, at the same time, induce proliferation in the reproductive system while being antiproliferative in the cardiovascular system. Data on the expression and regulation of the two ERs in different vasculopathies, and the identification of potential mechanisms behind estrogen's vasculoprotective action, could enable the development of tissue-selective estrogens for the prevention of restenosis and allograft arteriosclerosis.

The specific aims were:

1. To investigate the expression and regulation of ERα and ERβ in response to vascular injury using rat carotid denudation and cardiac transplantation models.

2. To try to differentiate between estrogen's beneficial vasculoprotective properties and its harmful side-effects with selective targeting of ERβ.

3. To compare and correlate the vasculoprotective properties of four different SERMs with natural estrogen *in vitro* and *in vivo*, concentrating particularly on SMC biology.

4. To investigate the interactions between natural estrogen and peptide growth factors in estrogen's

METHODS

1. Rat models for restenosis and allograft arteriosclerosis

Experimental animals. Female or male Wistar rats and specific pathogen-free inbred male DA (AG-B4, RT1[a]) and WF (AG-B2, RT1[u]) rats, purchased from the Laboratory Animal Center, University of Helsinki, Helsinki, Finland (I, II, IV) or from Harlan, Holland (III) were used for denudation and transplantation studies, respectively. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The investigation was approved by the Haartman Institute Ethical Committee for Animal Studies, and the permission to perform the experiments was granted by the Government of the County of Southern Finland. The rats that received estrogenic drugs were placed on a soy-free diet (Special Diet Services, Essex, UK) seven days before operation (I, III, IV); in experiment II, the rats received the basic diet (Altromin, Standard Diet, Chr. Petersen A/S, Ringsted, Denmark). Tap water was given ad libitum. The rats were anesthesized with chloralhydrate, 240 mg/kg i.p., and 0.25 mg/kg s.c. buprenorphine (Temgesic, Reckitt & Colman, Hull, UK) was used for peri- and post-operative pain relief.

Rat carotid and aortic denudation. Female rats were ovariectomized through abdominal incision seven days before denudation to restrain physiological estradiol production. The left common carotid artery (I) or the thoracic aorta (III, IV) was denuded of endothelium by an intraluminal passage of a 2 French (2F) Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Santa Ana, CA), which was introduced through the external carotid artery or the left iliac artery, respectively. The balloon was inflated with 0.2 ml of air and passed three times in the carotid artery or five times in the thoracic aorta. Thereafter, the external carotid artery, or iliac artery, was ligated and the wound was closed. At sacrifice, the artery was removed, a middle section was processed for histology, and the rest was snap-frozen in liquid nitrogen and stored at -70°C until used. In female rats, the uterus was also removed, weighed, and processed for histology.

Rat cardiac transplantation. Intra-abdominal heterotopic rat cardiac allografts were transplanted using a microsurgical technigue modified from Ono and Lindsey (Ono and Lindsey 1969). Syngeneic transplantations were performed from DA to DA rats and

allogeneic transplants from DA to WF rats. Ice-cold heparin-PBS was perfused through the vena cava inferior of the donor, and thereafter, caval vessels and pulmonary veins were ligated, and aorta and pulmonary artery were cut 3-5 mm above their origin. Donor heart was preserved in ice-cold PBS for 15 min. Then, donor heart aorta and pulmonary vein were anastomosed to recipient abdominal aorta and inferior vena cava between renal vessels and aorta bifurcation using 9-0 nylon suture. Total ischemic time was 45 ± 15 min, during which the donor heart was cooled with cold PBS gauze. The grafts started beating vigorously after circulation into the graft was established. The function of the graft was evaluated by abdominal palpation and all the grafts were beating at removal.

2. Drug regimens

Cyclosporine A (CsA) was used for base immunosuppression of allogeneic transplants. Fifty mg/ml of CsA infusion substance (Sandimmun; Sandoz Pharma AG, Basel, Switzerland) was dissolved in 200 mg/ml Intralipid (KabiVitrum, Stockholm, Sweden) to a final concentration of 1.0 mg/ml. Cyclosporine was administered daily s.c., 2 mg/kg/d for the first seven days and then 1 mg/kg/d. Whole blood CsA 24-hour trough levels were measured weekly with radioimmunoassay (Sandimmun-Kit; Novartis, Basel, Switzerland).

17β-estradiol (E2; Sigma, St Louis, MO) was dissolved in dimethyl sulphoxide (DMSO; Sigma) and 0.9% NaCl (Baxter, Vantaa, Finland), and administered s.c. using the following doses: 0.0025, 0.025, 0.25, and 2.5 mg/kg/d, in one injection per day. For long-term follow-up, a dose of 2.5 mg/kg/d was chosen, based on the dose responses. The treatment was initiated at the time of operation. Thereafter, the animals were weighed daily, and the dose was adjusted according to weight changes. Control animals received an equal volume of vehicle (DMSO, 0.9% NaCl, 1:1). Serum 17β-estradiol levels were measured with competitive radioimmunoassay (RIA) at the Laboratory of the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Helsinki, Finland. For *in vitro* studies, a 50 mM stock solution was prepared in DMSO and this stock solution was diluted in cell culture medium and used for the experiments at concentrations of 0.0006-50 μM. The concentration of DMSO in all *in vitro* experiments remained below 0.1%.

Genistein was generously provided by Dr. William Helferich (Michigan State University, East Lansing, MI) or purchased from Plantech (York, UK). It was dissolved in DMSO and

0.9% NaCl, and administered s.c. using the same doses as with E2. Genistein was also used for *in vitro* experiments and the solutions were prepared similar to E2.

SERMs. Tamoxifen (Sigma), raloxifene (Evista; Eli Lilly, Houten, The Netherlands), ospemifene (Hormos Medical Corporation, Turku, Finland), and fispemifene (Hormos Medical) were all dissolved in DMSO and 0.9% NaCl. The drugs were administered s.c. as a single daily injection with the following doses: 0.0025, 0.025, 0.25, 2.5, and 25 mg/kg/d. For long-term follow-up a dose of 2.5 mg/kg/d was used. The treatment was initiated at the time of operation. The animals were weighed daily, and the dose was adjusted according to changes in weight. Control animals received an equal volume of vehicle (DMSO, 0.9% NaCl, 1:1). Solutions for *in vitro* experiments were prepared as with E2.

ICI 182,780 (Faslodex; kindly provided by Tuula Ikonen, Zeneca Pharmaceuticals, Helsinki, Finland), a pure anti-estrogen, was dissolved in DMSO and 0.9% NaCl and administered s.c. as a single daily injection with the following doses: 0.0025, 0.025, 0.25, and 2.5 mg/kg/d. For long-term follow-up a dose of 2.5 mg/kg/d was used. Solutions for *in vitro* experiments were prepared as described above. The relative binding affinities (RBA) of all the compounds used in the study are given in Table 4.

Table 4. Relative binding affinities (RBA) of the different compounds to ERα and ERβ

Drug	RBA		References
	ERα	**ERβ**	
17β-estradiol	100	100	Kuiper et al. 1998
Genistein	4	87	Kuiper et al. 1998
Raloxifene	69	16	Kuiper et al. 1998
Tamoxifen	4	3	Kuiper et al. 1998
Fispemifene	1.1	0.55	Lauri Kangas, personal communication
Ospemifene	0.82	0.59	Qu et al. 2000
ICI 182,780	89	166	Wakeling et al. 1991, Kuiper et al. 1997

3. Quantitative RT-PCR

Total RNA isolation. Total RNA was isolated from snap-frozen E2- or vehicle-treated aorta specimens by the guanidium isothiocyanate method (Chomczynski and Sacchi 1987). After isolation and purification, RNA concentrations were measured by a spectrophotometer and the quality of RNA was confirmed by electrophoresis through 1% agarose gel.

Quantitative RT-PCR. Total RNA was isolated as described above. Six serial dilutions were made with an appropriate amount of total RNA from each group, mixed with either 10^6 or 10^7 molecules of synthetic template RNA containing a 46-nucleotide insert and reverse transcribed to cDNA in a mixture containing 1x RT buffer (Promega, Madison, WI), 300 mM dNTP (GeneAmp; Perkin Elmer, Foster City, CA), 10 pmol of antisense primer (IGF-1, TCTGTAGGTCTTGTTTCCTGCA; IGF-1R, GACGGACTACTACCGGAAAGG; PDGF-A, ATGTCACACGCCACGTACAT; PDGF-Rα, CACACTGAAGGTTCCGTTGAAG; PDGF-B, CACTACTGTCTCACACTTGCAGG; PDGF-Rβ, TCATAGGGTACATGTAGGGGG AT), 20 U RNasin (RNasin Ribonuclease Inhibitor; Promega), and 100 U of reverse transcriptase (M-MLV-RTase Reverese Transcriptase, RNase H Minus; Promega) at +37°C for 90 minutes. Two ml of cDNA mixture was supplemented with 10x PCR buffer (GeneAmp, Perkin Elmer), radioactive tracer, 10 pmol of antisense primer, 10 pmol of sense primer (IGF-1, GGAAAATCAGCAGTCTTCCAAC; IGF-1R, TTCCGATGATCTCCAGGAAG; PDGF-A, GACAAACCTGAGAGCCCATG; PDGF-Rα, GAGAAGATTGTGCCGCTGAGT; PDGF-B, CTGAGCTGGACTTGAACATGAC; PDGF-Rβ, TCGTCCTCAACATTT CGAGC), and 2.5 U of *Taq* polymerase. The samples were heated to +95°C for 5 minutes and cycled 40 times (+94°C for 30 seconds, +60°C for 30 seconds, and +72°C for 1 minute). The samples were electrophoresed through 2% agarose gel and incorporated radioactivity was quantitated. Accession numbers are given in the original publication (IV).

4. *In situ* hybridization

Probe Preparation. The complementary RNA probes were synthesized according to the Manufacturer's (Promega) directions in the presence of ^{35}S-UTP (Amersham Pharmacia) by using the following cDNA fragments as templates. A 400-bp fragment (from the 5' untranslated region, starting nucleotide sequence GAATTC, ending nucleotide sequence CTACGT) of rat ERβ cDNA and a 200-bp fragment (from the 3' untranslated region, F-domain region, starting nucleotide sequence ATGGGA, ending nucleotide sequence TAGCAG) of the rat ERα cDNA were subcloned to pBluescript II KS (+) (Stratagene, La Jolla, CA) vector and used for the production of corresponding antisense and sense cRNA probes. RNA probes transcribed from opposite strands of the same plasmid template were adjusted to the same specific radioactivity.

In situ **hybridisation.** Rat carotid arteries and heart grafts were removed at different time points post operation, fixed in 3% paraformaldehyde solution for 4 hours, transferred to saline, and processed for paraffin embedding. Human heart allograft endomyocardial biopsies came from the pathology files of the Transplantation Laboratory, and the permit to use the biopsies in this study was granted by the Helsinki University Central Hospital Ethical Committee. Uterus and bladder specimens were chosen for control tissues, as uterus is known to express predominantly ERα and bladder ERβ (Kuiper et al. 1997).

Serial sections of the different specimens were made under RNase free conditions. To ensure that the different specimens were comparable, sections from each group and the control tissues were placed on the same silanized microscopy slide and hybridized in identical conditions, either for ERα or ERβ. After deparaffinization and rehydration, the sections were denatured in 0.2 M HCl, heat-denatured in 2x standard saline citrate (SSC) at +70°C, and treated with proteinase K (1 μg/ml). The sections were then post-fixed with 4% paraformaldehyde, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, and dehydrated and air-dried. The slides were hybridised with antisense and sense RNA probes at +60°C overnight, washed in 4x SSC, treated with RNase A (20 μg/ml), and washed sequentially in SSC solutions with 1 mM DTT. Finally, the slides were rinsed in 0.1x SSC with 1 mM DTT, dehydrated in graded ethanols, and air-dried. Then, the slides were dipped into autoradiography emulsion (NBT-3; Eastman Kodak, Rochester, NY), exposed for 7-14 days, and developed, counterstained, dehydrated, and mounted with Permount. The number of grains was calculated from three randomly-selected areas per slide using 100x objective and oil immersion technique.

5. Western blotting

Snap-frozen aortas were crushed and homogenized with Ultra-Turrax (Janke & Kunkel, Staufen, Germany) in 1 ml of buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L NaF). The homogenate was supplemented with 1% Triton X-100, 2 mmol/L PMSF, 100 units of aprotinin, and 10 mmol/L benzamidine, and after incubation at +4°C for 1 hour, the lysate was clarified by centrifugation. The supernatant was split and incubated with 10 µl of appropriate rabbit polyclonal antibodies for IGF-1, IGF-1R, PDGF-AA, PDGF-Rα, PDGF-BB, and PDGF-Rβ, and further with 50 µl of Protein A sepharose suspension in lysis buffer (+4°C, 1 hr). Captured immunocomplexes were washed and

separated using standard SDS-PAGE (15% polyacrylamide for receptors and 5% for ligands). The gel was transferred to a nitrocellulose membrane and blocked with 20 mmol/L NaH_2PO_4, 80 mmol/L Na_2HPO_4, 100 mmol/L NaCl, 0.1% Tween-20, and 5% skimmed milk powder (+4°C, overnight). Then, the membrane was incubated with corresponding antibodies (Santa Cruz Biotechnology) in blocking buffer (RT, 1 hr). After washing in blocking buffer (without 5% skimmed milk powder; RT, 30 min), the membrane was incubated with secondary antibodies (RT, 1 hr), washed again and treated with ECL (Amersham International Plc., Little Chalfont, England). The signal was visualized by exposure to Kodak X-OMAT-AR film. Signal intensity was measured with Macintosh NIH image software (National Technical Information Service, Springfield, VI). Details for the antibodies are given in the original publication (IV).

6. Histological and immunohistological evaluation

Morphometry. For evaluation of vascular morphological changes, carotid and aortic specimens were fixed in 3% paraformaldehyde (pH 7.4) solution for 4 hours, transferred to saline, and processed for paraffin embedding. Two μm thick cross sections were made and stained with Mayer's hematoxylin and eosin. The absolute number of cells in the intimal, medial, and adventitial layers was calculated from paraffin cross sections using 400x magnification. Intimal (inside *lamina elastica interna*) and medial areas (between *lamina elastica interna* and *lamina elastica externa*) and vessel lumen were quantitated with Macintosh NIH image software and the intimal/medial area ratio was calculated from these values. For uterus histology, the specimens were processed as above, and specimens from vehicle-treated rats were compared blind to specimens from rats that were treated with the different drugs for 7 or 28 days.

Quantitation of cell proliferation *in vivo*. Proliferating cells were labeled with intravenous injection of 300 μl of 5-bromo-2'-deoxyuridine (BrdU; Pharmacy of Helsinki University Central Hospital, Helsinki, Finland), 3 hours before sacrifice. Incorporation of the labeling reagent was visualized by immunohistochemical staining of paraffin cross sections, using a commercial immunoperoxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) as described below. Finally, the absolute number of positively-stained nuclei was counted microscopically using 400x magnification.

Immunohistochemistry. Serial frozen sections or cultured rat SMC 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 air-dried. Sections from paraformaldehyde-fixed paraffin-embedded specimens were deparaffinized before staining. In case of polyclonal primary antibodies, the sections were sequentially incubated with 1.5% normal goat serum (Vector Laboratories), primary antibody (+4°C, overnight), biotinylated goat anti-rabbit antibody, avidin-peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories), 0.1% hydrogen peroxide, and 3-amino-9-ethylcarbazole (AEC; Sigma), with intervening washes with Tris-buffered saline. Finally, the slides were counterstained with hematoxylin and coverslips were mounted. With monoclonal primary antibodies, the slides were incubated with 1.5% normal horse serum (Vector Laboratories) and a mouse monoclonal primary antibody (+4°C, overnight). The subsequent steps were performed as above, except that biotinylated horse anti-mouse secondary antibody was used. With IGF-1 and IGF-1R antibodies, the sections were first incubated with 5% normal horse serum (Vector Laboratories) and thereafter with a primary antibody (+4°C, overnight), secondary antibody, avidin-alkaline phosphatase complex (Vectastain ABC-AP Kit; Vector Laboratories), and alkaline phosphatase substrate (Vector Red Alkaline Phosphatase Substrate Kit I; Vector Laboratories). The antibodies used are presented in Table 5.

To establish the specificity of the ER antibodies to rat and human ER subtypes, their reactivity to rat and human uterus and bladder were investigated. Additional controls for polyclonal primary antibody staining were performed using 10- to 20-fold molar excess of neutralizing synthetic peptides to the corresponding antibodies, and by omitting the primary antibody. Monoclonal primary antibody stainings were controlled by replacing the primary antibody with irrelevant antibody (clone DAK-GO1; DAKO). None of the control stainings showed any immunoreactivity.

Table 5. Antibodies used for immunohistochemistry

Species	Specificity	Clone/code	Antigen	Source	Dilution
Mouse, mono-clonal	BrdU	M 0744	Bromodeoxyuridine conjugated to bovine serum albumin	DAKO, Glostrup, Denmark	1:20
	ERα	M 7047	Recombinant human ER protein	DAKO	1:50
		NCL-ER-6F11	Recombinant full-length human ERα protein	Novocastra, Newcastle upon Tyne, UK	1:40
Rabbit, poly-clonal	ERβ	PAI-310	A synthetic peptide corresponding to the C-terminal amino acid residues 467-486 of rat ERβ	Affinity Bioreagents, Golden, CO	1:200
		PAI-311	A synthetic peptide corresponding to the N-terminal amino acid residues 55-70 of rat/mouse ERβ	Affinity Bioreagents	1:600
	IGF-1	AFP4892898	Human IGF-1 protein	National Institute of Digestive and Kidney Diseases, Bethesda, MD	1:2000
	IGF-1R	sc-712	A synthetic peptide corresponding to the N-terminal amino acid residues 31-50 of human IGF-1R	Santa Cruz Biotechnology, Santa Cruz, CA	1:200
	PDGF-AA	ZP-214	Purified recombinant human PDGF-AA protein	Genzyme, Cambridge, MA	1:100
	PDGF-Rα	sc-338	A synthetic peptide corresponding to the C-terminal amino acid residues of human PDGF-Rα	Santa Cruz Biotechnology	1:100
	PDGF-BB	ZP-215	Purified recombinant human PDGF-BB protein	Genzyme	1:100
	PDGF-Rβ	sc-339	A synthetic peptide corresponding to the C-terminal amino acid residues of human PDGF-Rβ	Santa Cruz Biotechnology	1:100
	Factor VIII	A0082	Von Willebrand Factor isolated from human plasma	DAKO	1:200

ERα and ERβ stainings were evaluated semiquantitatively by two different methods; separately from the intimal, medial, and adventitial layers of the vessels, or from the arteries, veins, myocardium, and inflammatory cells of the cardiac allografts. The intensity of staining was graded as follows: 0/- = no visible staining; + = weak staining; ++ = moderate staining; +++ = intense staining. The frequency of positive cells was expressed as the fraction of positively stained cells out of total: 0 = 0-10%; 1 = 15-35%; 2 = 40-60%; 3 ≥ 65%. Growth factor immunostaining was graded separately from intimal, medial, and adventitial layers of the aorta as follows: - = no visible staining; (+) = few cells with very faint staining; + = some cells with weak staining; ++ = moderate staining with multifocal expression; +++ = intense staining throughout the vessel compartment. With BrdU and factor VIII, the results were expressed as positive cells per cross section. All gradings were made blinded of the type of specimens.

7. Cell culture studies

Rat primary smooth muscle cell cultures. Primary vascular SMC were isolated from 9 to 11-day old DA (AG-B4, RT1[a]) rat aortas using a method modified from Thyberg et al. (Thyberg et al. 1983). The aortas were opened longitudinally and the endothelial layer was scraped off. Then, the adventitia and media were separated, and the medial layer was digested with 0.1% collagenase and DNAse in PBS at +37ºC for 30 minutes. The cells were centrifuged, suspended in culture medium, and thereafter, allowed to attach to plastic flasks. Primary cells were used at passages 10-15 for the experiments. For identification, the cells were grown until 70% confluency on glass slides, serum-starved for 24 hours and then stimulated with 10% FCS or left unstimulated for another 24 hours. All cells expressed SMC α-actin, and furthermore, 80% of the serum-starved and FCS-stimulated SMC expressed ERβ while only 1% of the serum-starved and none of the FCS-stimulated SMC expressed ERα.

The cells were subcultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The medium for experiments with estrogenic compounds consisted of DMEM without phenol red (BioWhittaker, Cambrex Bioproducts, Verviers, Belgium) and 5% Dextran charcoal-treated Fetal Bovine Serum (DCC-FBS; HyClone, Logan, UT), to exclude the estrogenic effects of phenol red in the medium or endogenous estrogens in the serum. *In vitro* studies were done in triplicate and repeated three times.

[3]H-TdR-incorporation. The cells were seeded on 96-well plates (5,000 cells/well) in DMEM supplemented with 5% FCS. The next day the growth medium was changed to serum-free and phenol red-free medium containing 0.5% bovine serum albumin (BSA). Following 48-hour serum starvation, proliferation was induced with either with PDGF-B (20 ng/ml, Upstate Biotechnology, Lake Placid, NY) or 5% DCC-FBS, and simultaneously, the serially-diluted drugs and ^{3}H-TdR (Amersham Pharmacia, Amersham, UK) were added to the wells, and incubation was continued for 24 or 48 hours. The amount of incorporated, radioactively labeled thymidine to the DNA, which corresponds to the amount of DNA synthesis in each well, was measured using 10% trichloroacetic (TCA) precipitation and counted separately from each well using a liquid scintillation counter (RackBeta; Wallac, Turku, Finland).

Smooth muscle cell migration. SMC migration was quantitated using Transwell culture chambers (Costar, Cambridge, MA) where the upper and lower culture chambers are separated by a polycarbonate filter with 8-μm pores. The chambers were first coated with collagen (20 μg/ml; Rat Tail Collagen, Type 1; Upstate Biotechnology, Lake Placid, NY) at +4ºC for 24 hours. Primary rat aortic SMC (50,000 cells/chamber) were seeded in the upper chamber in phenol red-free DMEM containing 0.5 % BSA, and PDGF (60 ng/ml) was added to the lower chamber, and the cells were allowed to adhere to the filter for 60 minutes. The serially-diluted drugs were added to the upper chamber, and after 24-hour incubation at +37ºC, the filters were removed, fixed with methanol, and stained with hematoxylin. The cells that had migrated on the lower side of the filter were quantitated by counting specific cross-sectional fields with a light microscope using 400x magnification.

8. Tissue culture studies

Aortic explant culture. Aortic explants were prepared from ovariectomized female Wistar rats that were denuded of endothelium on day –2. The rats were treated with the compounds at the dose of 2.5 mg/kg/d s.c. prior to sacrifice. Experiment media consisted of phenol red-free DMEM supplemented with 10 % DCC-FBS. At sacrifice, a small proximal part of each aorta was prepared for histology and vessel morphology was determined as described above. The rest of the aortas, including all layers of the vessel, were opened longitudinally and explants measuring 1x1 mm were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, England). The explants were placed individually into the wells of a flat-bottomed 96-well plate (Nunc, Roskilde, Denmark) with 20 µl basal medium to keep the explants damp. Thereafter, they were allowed to adhere to the tissue culture plates in a +37˚C incubator for 2 hours. Two hundred µl of experiment medium with serially-diluted drugs was then added to each well**.**

Quantitation of explant outgrowth. Each well was observed after 24 and 48 hours of culture and counted as positive for "sprouting" if one or more cells had grown out of the explants. The distance that the leading edge of migrating cells in each well had travelled from the explant was measured using a calibrated graticule (Olympus, Tokyo, Japan). To quantitate proliferating cells, the explants were pulsed with ^{3}H-TdR on day 0. On day 2 the outgrowing cells were detached from the wells with trypsin and the explants were digested with pepsin (Merck, Darmstadt, Germany) at +37˚C for 50 minutes. Then, the content of each well was

harvested with a Dynatech Harvester (Dynatech Labs, Sussex, UK) and mixed with OptiScint Hisafe (LKB-Wallac, Turku, Finland). Radioactivity was measured with a Rackbeta liquid scintillation counter.

9. Statistical analysis

All data are given as mean ± SEM. Significance of the observed differences between different groups was tested either with one-way ANOVA followed by Fisher's PLSD test, or in the case of unequal variances or only two groups, with Student's unpaired t test. Prior to these tests, normal distribution of the data was tested. In addition, linear regression analysis, preceded by logarithmic transformation, was used for dose-response analyses. All analyses were performed using Statview 4.1 software (Abacus Concepts, Berkeley, CA). Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

1. Estrogenic effects in the rat carotid and aorta denudation injury models (I, III-IV)

Mechanical injury was inflicted to rat carotid artery or aorta with a balloon catheter, which resulted in complete loss of the luminal endothelium. The first cells appeared in the intima after day 3. Proliferation peaked in the media at 3 days and in the intima at 7 days, and this was followed by a rapid increase in the number of intimal nuclei and intimal area within 7 to 14 days post-operation. Thereafter, intimal growth subsided and further increase was small. The number of medial nuclei remained unaltered throughout the time interval investigated.

Although estrogen's vasculoprotective properties on the injury response have been previously demonstrated in the rodents (Foegh et al. 1994, Sullivan et al. 1995, Chen et al. 1996), no comprehensive dose-response studies exist, which is why those were performed first. Effective therapeutic doses for long-term follow-up were chosen based on the dose-responses. On day 7, estradiol treatment dose-dependently reduced the number of intimal nuclei in both the carotid artery (r=0.864) and aorta (r=0.693). With the highest dose (2.5 mg/kg/d, s.c.) the intimal nuclei number remained at the level of normal artery.
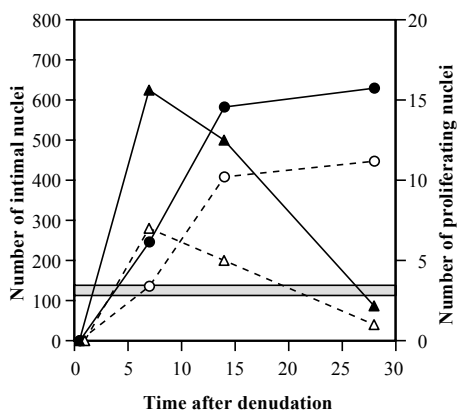
Figure 9. *Effects of 17β-estradiol (E2) treatment (2.5 mg/kg/d) on the ovariectomized female rat aorta at 0, 7, 14, and 28 days after denudation injury are presented in the upper figure. Mean number of nuclei in the vessel intima in the vehicle (closed circle) and E2 (open circle) groups, and mean number of proliferating (BrdU-positive) nuclei in the vehicle (closed triangle) and E2 (open triangle) groups are shown, n=6-10. The grey rectangle represents the number of nuclei (endothelial cells) in a normal female rat aorta.*

Representative microphotographs of vehicle (left) and E2-treated (right) female rat aorta at 28 days after denudation injury are presented in the lower figure. The arrows indicate lamina elastica internae. Lumen facing up, x400.

Vehicle   17β-estradiol
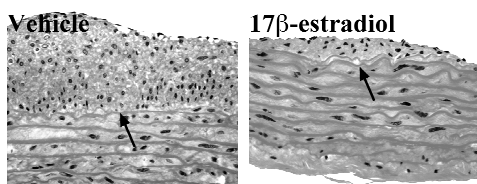
Long-term treatment up to 28 days (2.5 mg/kg/d) resulted in approximately 30%-50% reduction in the number of intimal nuclei (Fig. 9) and intimal area of the aorta, and 50%-70% reduction in the number of proliferating (BrdU-incorporating) nuclei (Fig. 9). Concomitantly, a 4-fold increase in reendothelialization was observed. Serum estradiol levels with the highest dose (2.5 mg/kg/d) were approximately 10 times higher than the levels in a normal woman and 100 times higher than in a normal female rat.

Treatment of male rats with 0.25 mg/kg/d of E2, s.c., resulted in significant inhibition in the number of intimal nuclei, intimal area, and proliferating nuclei on day 7, and the changes were comparable to those observed in female rats.

2. Estrogen receptor expression in the rat vascular wall after endothelial injury (I)

The expression and localization of ERα and ERβ mRNA in male rat carotid artery at different time points after endothelial denudation injury were investigated by *in situ* hybridisation (Fig. 10). Both ERα and ERβ transcripts were expressed at low levels in the normal carotid artery. ERβ was upregulated markedly in the media, and especially in the neointima, within 3 to 28 days post-injury, peaking on day 7, while expression of ERα remained unchanged.
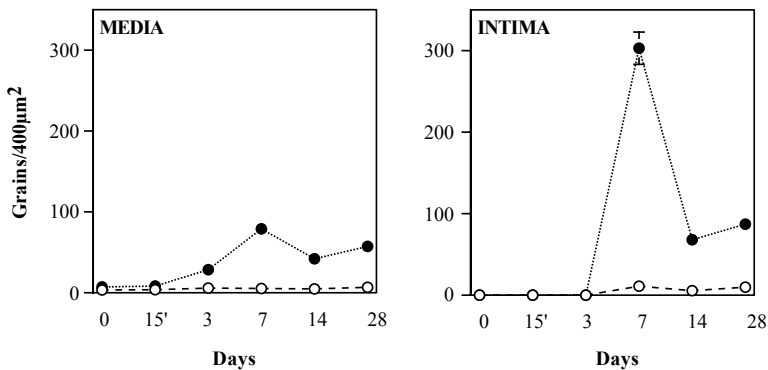
**Figure 10.** *Expression of ERα (open circle) and ERβ (closed circle) mRNA in male rat carotid artery at different time points after denudation injury determined by in situ hybridisation. Data are given as mean±SEM, n=3 and three countings per section.*

In situ hybridisation and immunohistochemistry demonstrated that ERβ mRNA and protein were colocalized with the SMCs in the media and neointima, over 90% of which were positive for α-actin.

3. Estrogen receptor expression in rat and human cardiac allografts (II)

The localization and intensity of the expression of ERα and ERβ mRNA and protein were investigated by *in situ* hybridisation and immunohistochemistry in rat male-to-male cardiac allografts and human male-to-male heart allograft endomyocardial biopsies with various degrees of acute or chronic rejection.

Almost no ER mRNA was seen in the normal (non-transplanted) DA rat heart arteries, veins, heart muscle, or inflammatory cells; however, ERβ protein was present. ERβ mRNA and protein were prominently expressed in the rat allogeneic (under rejection) and syngeneic graft (subject to reperfusion injury) vessels and stroma, whereas ERα mRNA and protein were present in small amounts only. The vessels, stroma, and inflammatory cells in human endomyocardial biopsies stained positively for ERβ protein, whereas some ERα was seen only in the myocardium and inflammatory cells. When correlated to the expression levels of ERs in uterus and bladder, the former of which expresses mainly ERα and the latter ERβ (Kuiper et al. 1997), it was observed that the expression of ERβ in the grafts was close to its expression levels in bladder epithelium, whereas the expression of ERα was – even at its highest - only 30%-50% of the levels in uterine endometrium. Interestingly, the expression of ERβ in the grafts showed only a weak correlation with the intensity of rejection.

4. Differentiation between estrogen's vasculoprotective and uterotrophic effects (I)

To investigate if estrogen's vasculoprotective properties could be differentiated from its gender-related effects, we compared the effects of 17β-estradiol (which binds both ERs with high affinity) and the phytoestrogen genistein (which binds ERβ 20 times stronger than ERα) on neointimal thickening and uterine growth following carotid artery denudation injury in ovariectomized female Wistar rats. To exclude the effect of food-derived (phyto)estrogens, the rats were placed on soy-free diet at the time of ovariectomy.

Both E2 ($r^2=0.746$) and genistein ($r^2=0.946$, p=0.5 as compared to E2) had a dose-dependent inhibitory effect on the number of intimal nuclei within the dose range from 0.0025 to 2.5 mg/kg/d. Moreover, both compounds similarly reduced the number of replicating (BrdU-incorporating) nuclei in the neointima. *In vitro,* in the supraphysiological dose range, both compounds equally inhibited the PDGF-induced ^{3}H-TdR-incorporation (DNA synthesis) and migration of cultured rat vascular SMC.

However, only E2 ($r^2=0.954$) dose-dependently increased the weight of the uterus and induced typical estrogen-related morphological changes, such as hyperplastic endometrium, stromal thickening, and mild stromal inflammation, while genistein had no effect ($r^2=0.096$, p=0.0003 as compared to E2).

5. SERMs' vasculoprotective properties in the injured rat vascular wall (III)

All SERMs studied, as well as the pure anti-estrogen ICI 182,780, dose-dependently (from 0.0025 to 25 mg/kg/d s.c.) inhibited the number of intimal nuclei at 7 days after aorta denudation injury in ovariectomized female rats. Tamoxifen, raloxifene, and ICI 182,780 also reduced SMC proliferation (the number of BrdU-incorporating nuclei) in the intimal layer, similarly to E2. When treated with the most effective dose (2.5. mg/kg/d) for 28 days, only tamoxifen and ospemifene reduced the number of intimal nuclei and intimal area, and the effect was comparable to E2. Tamoxifen (p=0.0002), raloxifene (p=0.03), ospemifene (p=0.04), and fispemifene (p=0.0006) (2 to 3-fold), but not ICI 182,780, significantly enhanced reendothelialization at 28 days post-injury.

In order to study the reversibility of the vasculoprotective effect, additional groups of rats were treated with the compounds for 14 days, whereafter the compounds were replaced with vehicle for another 14 days. No rebound effect was observed with E2, tamoxifen, or ospemifene, and furthermore, a smaller neointima size than with continuous treatment was achieved with raloxifene, fispemifene, and ICI 182,780. Interestingly, with RA the number of factor VIII-expressing (endothelial) cells in the luminal side of the artery doubled after discontinuation of the treatment.

Finally, only E2 dose-dependently increased the weight of the uterus and induced typical estrogen-related morphological changes, while only weak (tamoxifen, ospemifene, fispemifene) or no (raloxifene, ICI 182,780) such effect was observed with the SERMs.

6. Estrogenic effects in the rat vascular SMC and aortic explant cultures (I, III)

Estradiol inhibited DNA synthesis (^{3}H-TdR incorporation) of cultured rat primary SMC. The inhibitory effect was weak or neutral in the physiological dose range and became significant only at micromolar concentrations. Estradiol also had a dose-dependent inhibitory effect on the PDGF-induced migration of SMC at concentrations above 10^{-8} M. All SERMs had estrogenic effects on SMC DNA synthesis and, overall, their antimitogenic effects were superior to estrogen. All compounds, excluding fispemifene, inhibited SMC migration as well. In the aortic explants, estradiol reduced ^{3}H-TdR incorporation and migration of SMC already at nanomolar concentrations. All SERMs were anti-mitogenic, and all but fispemifene were also anti-migratory. Interestingly, the pure antiestrogen ICI had opposite effects in the SMC and explant cultures: it was antimitogenic to SMC but had neutral effects on the explant cultures.

7. Regulation of growth factor expression in the rat vascular wall by estrogen (IV)

Growth factor mRNA and protein expression and localization in the denuded male rat aorta were investigated at 15 min, 3, and 7 days post-injury. Quantitative RT-PCR and Western blotting showed significant upregulation of IGF-1, PDGF-Rα, and PDGF-B mRNA and protein after injury, concomitantly with the increase in proliferative SMC in the developing neointima. Administration of estradiol completely abolished IGF-1 mRNA and protein expression whereas it had no effect on IGF-1R. It also downregulated PDGF-Rα, and PDGF-B, but had no effect on PDGF-Rβ. Estrogen therapy reduced immunoreactivity for IGF-1, PDGF-A, PDGF- Rα, and PDGF-B, while no changes were seen with respect to IGF-1 or PDGF- Rβ.

DISCUSSION

Estrogen is a pleiotropic steroid hormone with varied effects on cell proliferation, differentiation, and growth in different parts of the body. Previously, it was difficult to understand how estrogen may have growth-promoting effects in some tissues, such as the uterus and breast, and growth-inhibitory effect in other tissues, including the cardiovascular system. The paradigm, however, was potentially unravelled by the discovery of a second ER, ERβ, in 1996, and currently, many of the contrasting effects of estrogen may be explained by the tissue- and/or cell type-specific expression and regulation of ERα and ERβ. The mechanisms behind estrogen's vasculoprotective properties are still incompletely understood.

1. Experimental models for studying restenosis and allograft arteriosclerosis

Preclinical experimental studies on the development of restenosis or allograft arteriosclerosis and their intervention in man can only be carried out in an *in vitro* environment with human cell lines or tissues. Therefore, experimental models in animals are needed for the *in vivo* studies. Nonhuman primates, such as baboon, have the closest resemblance to human, but they are expensive, difficult to handle and maintain in the laboratory, and not readily available.

Rat carotid artery and aorta denudation injury models have been widely used to study the development of restenosis and the coexistent SMC proliferation and migration in the vascular wall (Clowes et al. 1983a, Clowes et al. 1983b, Reidy et al. 1983, Myllarniemi et al. 1997). The models differ from the human setting in some respects. Rat artery has a much thinner endothelium and no natural intima (Sims 1989), and the injury in the rat is induced in a healthy artery whereas the human vessel is already diseased. Also, the rat model does not take into account several risk factors, such as hypertension or genetic susceptibility. Moreover, human but not rat angioplasty requires antiplatelet/anticoagulant therapy. Compared to baboon, the injury response is stronger and reendothelialization slower in rat carotid artery (Du Toit et al. 2001). However, the rat model enables us to study the events in the vascular wall in an *in vivo* environment, which makes it superior to all *in vitro* experiments. With respect to this study, the beneficial vascular properties of estrogen have been shown both in rodents and in humans (Stampfer et al. 1985, Grady et al. 1992, Foegh et al. 1994, Chen et al. 1996). Moreover, human ERβ gene shows approximately 89% identity to rat ERβ (Enmark et al. 1997).

It has recently become apparent that most of the cells contributing to neointimal hyperplasia in restenosis as well as in allograft arteriosclerosis derive possibly from recipient-derived multipotent somatic stem cells and not from the vascular media (Hillebrands et al. 2000, Saiura et al. 2001, Shimizu et al. 2001, Hu et al. 2002, Sata et al. 2002, Tanaka et al. 2003). Therefore, the use of cultured medial SMC or SMC lines alone may not give a correct picture of the events in the injured vascular wall. Previous experiments have shown that intimal injury *in vivo* activates aortic explant outgrowth (sprouting) *in vitro* (Grunwald and Haudenschild 1984, Aavik et al. 2002), and that remarkable upregulation of sprouting occurs within 2-14 days post-denudation. Our unpublished data show that the majority (>75%) of the outgrowing cells express SMC α-actin, and numerous cells show features of undifferentiated endothelial- and myofibroblast-like cells. Due to the complexity of the vascular tissue, the aortic explant model shows more variability than the cell culture models, and thus, demonstration of statistical significance may be more difficult. However, the comparability of the specimens was ensured by morphological analysis of histological specimens extracted from each vessel. Although further identification of the outgrowing cells needs to be done, we may conclude that the aortic explant model allows us to evaluate the *in vivo* injury-induced changes in the vessel wall in an *ex vivo* setting, and it enables us to separately quantitate the proliferative and migratory responses without excluding the influx of precursor cells. This study (III) further demonstrates that the aortic explant model more reliably resembles the situation in the *in vivo* vessel: E2 inhibited clearly, and at lower concentrations, explant outgrowth, migration and proliferation in the explant model compared to cultured SMC. Also, ICI had contrasting effects in the explants vs. SMC: it was inhibitory on SMC proliferation *in vitro*, but not in the aortic explants *ex vivo*, which is similar to the *in vivo* setting.

2. Contribution of the two estrogen receptors on estrogen's vasculoprotective properties

Estrogen's effects in the body are mediated via both a rapid nongenomic pathway and a genomic pathway that requires the transcriptional regulation of target genes. Most of its vasculoprotective properties, excluding vasomotor effects, are thought to occur via the genomic pathway. Both ERs have been detected in cultured vascular SMC of cynomolgus monkeys (Register and Adams 1998) and in rat and human arteries (Lindner et al. 1998, Hodges et al. 2000). However, the relative contribution of the two ERs in mediating estrogen's actions in the vasculature remains unclear.

Knock-out mice, where either or both of the ERs have been deleted, have brought some insight in this issue. The finding that estrogen's vasculoprotective properties were preserved in ERα-/- mice (Iafrati et al. 1997) suggested that estrogens vascular effects are mediated via ERβ rather than ERα. This encouraged us to further study the role of ERβ in the injured vascular wall. ERβ clearly predominated over ERα in the rat carotid artery after denudation injury, colocalizing with the SMC in the media, and particularly in the neointima (I). ERβ was also predominantly expressed in rat and human cardiac allograft arteries, veins, myocardium, and inflammatory cells, whereas ERα remained at low level only (II). Furthermore, estrogen's vasculoprotective effect could be differentiated from its uterotrophic effect with specific targeting to ERβ (I). Predominant expression of ERβ in the normal and injured vascular tissue has, thereafter, been supported by studies in baboon and man (Hodges et al. 2000, Aavik et al. 2001). Contrary to these findings, ERα was shown to be upregulated in rabbit cardiac-aortic allografts (Lou et al. 1998a). However, ERα mRNA was increased only 3-fold, which is remarkably less than the nearly 40-fold increase in ERβ mRNA in the injured rat artery (I). Moreover, the expression of ERβ was not determined in that study. Finally, estrogen's vasculoprotective properties were preserved in ERβ knock-out mice (Karas et al. 1999), but not in double knock-out mice (Karas et al. 2001), which suggests that, unless additional ERs exist, either of the two ERs is sufficient to protect against vascular injury. However, the mice in these studies did not develop *any* neointima and the analysis was based on modest changes seen in the media only, which differs markedly from the case in other rodents, primates, or humans. Furthermore, in one study, there was practically no injury-response at all (Pare et al. 2002). Thus, we cannot draw a conclusion on the relative roles of the two ERs in vasculoprotection based solely on the knock-out mouse data.

An alternative, or complementary, way to approach the contribution of the two ERs in estrogen's vasculoprotection would be ER subtype-selective ligands. Until recently, genistein was the most ERβ-selective compound available with approximately 20-fold affinity difference for ERβ vs. ERα (Kuiper et al. 1998). Treatment of ovariectomized rats with genistein, inhibited neointima formation and the proliferation and migration of vascular SMC equally to E2, while only E2 had a dose-dependent uterotrophic effect (I). Thus for the first time, estrogen's vasculoprotective effect was differentiated from its uterotrophic effect with special targeting to ERβ. Genistein can also exert antimitogenic effects through inhibition of tyrosine kinases (Akiyama et al. 1987, Epstein et al. 1997, Nelson et al. 1997, Schonherr et al.

1997, Takahashi et al. 1997). However, this was not the case in this study since genistein's tyrosine kinase inhibition has been reported to require relatively high (>10 μM) concentrations of genistein, whereas nanomolar concentrations are sufficient to exert significant ER-mediated effects (Makela et al. 1994, Kuiper et al. 1998). In our study, the inhibitory effects on vascular SMC proliferation *in vitro* occurred already at concentrations of 1 μM and on SMC migration at 0.1 μM. Furthermore, the *in vivo* doses were estimated to result in nanomolar serum concentrations (Santell et al. 1997).

Despite the relatively good ER-subtype selectivity of genistein, it still has ERα agonist activity (Kuiper et al. 1998). Only recently, highly ER subtype-selective compounds with no cross-reactivity have been described (Meyers et al. 2001, Harris et al. 2002, Sun et al. 2002, Harrington et al. 2003, Harris et al. 2003). Current understanding, gained from the knock-out mouse and ER subtype-selective estrogen studies, is that ERα is crucial to the uterotrophic, anorectic, lipid-lowering, and bone-sparing actions of estrogen (Harris et al. 2002), whereas ERβ seems to have immunomodulatory or anti-inflammatory, but not uterotrophic or mammotrophic activity (Harris et al. 2003). However, as the knock-out data on the vascular injury response may not be reliable and no data, besides with genistein (I), have yet been published on ER subtype-selective ligands, the concept in the vasculature remains unclear. Interestingly, recent *in vitro* studies suggest that estrogen's inhibitory effect on SMC proliferation and the concomitant mitogen-activated protein kinase inhibition is mediated via ERβ (Geraldes et al. 2003, Watanabe et al. 2003). This clearly supports our hypothesis.

It could also be questioned, if the estrogenic compounds' effects on neointima formation and SMC kinetics are mediated via ERs or via some alternative pathways. Both promoting and contradictory data exist. Bakir and coworkers have demonstrated that estrogen's vasoprotective effect in the injured rat carotid is blocked with a pure anti-estrogen ICI 182,780 (Bakir et al. 2000), which suggests that the effects are receptor mediated. ICI also blocked the inhibitory effect of E2 on human vascular SMC (Dubey et al. 2000) and, furthermore, vascular injury response was totally abolished in the ER double knock out mice (Karas et al. 2001). On the other hand, ICI, which is defined as a pure antiestrogen with no agonist activity, may also have anti-mitogenic activity on vascular SMC *in vitro* and *in vivo* (III; Bakir et al. 2000). ICI has been shown to decrease DNA synthesis in neonatal rat cardiac myocytes via an ER-independent pathway by regulating cell cycle proteins and extracellular signal-regulated kinase (Mercier et al. 2003). Furthermore, in an ER-negative ovarian cancer cell line, ICI

dose-dependently inhibited cell proliferation and induced apoptosis (Ercoli et al. 1998). Potential ER-independent mediators of estrogen action in the vasculature might include estrogen-related receptors (ERR) (Sladek et al. 1997, Susens et al. 2000, Sumi and Ignarro 2003) and plasma membrane estrogen binding sites (Chambliss et al. 2000, Russell et al. 2000, Chambliss et al. 2002, Li et al. 2003). Also, estradiol metabolites, such as 2-hydroxyestradiol and 2-methoxyestradiol, which have minimal affinity for the ERs, have been demonstrated to inhibit SMC and cardiac fibroblast growth via an ER-independent pathway *in vitro*, and even more potently than estradiol alone (Nishigaki et al. 1995, Dubey et al. 1998, Dubey et al. 1999). Thus, ER-independent mechanisms may also play some role in, and add to, the vasculoprotective effects of estrogen and related compounds.

3. Estrogen's interactions with growth factor signalling

Peptide growth factors are important in the development of vascular neointimal hyperplasia as they are upregulated in the vessel wall and induce the proliferation and migration of vascular SMC (Majesky et al. 1990, Cercek et al. 1991, Miano et al. 1993), and as blocking of their action inhibits neointimal thickening (Ferns et al. 1991b, Hayry et al. 1995, Myllarniemi et al. 1997).

The interplay of estrogen with different growth factors is complex and varies in different tissues. First, estrogen-induced proliferation in the uterus is accompanied by increased expression of polypeptide growth factors or their receptors, such as IGF-1, EGF, and TGF-β (Murphy et al. 1987, Huet-Hudson et al. 1990, Nelson et al. 1992, Das et al. 1994). Second, these same growth factors can activate estrogen receptors in a ligand-independent manner (Ignar-Trowbridge et al. 1992, Aronica and Katzenellenbogen 1993). Mitogen-activated protein kinase (MAPK), activated by growth factors such as IGF-1, PDGF, and EGF through their cell surface receptors, is capable of phosphorylating the AF-1 domain of ERα and ERβ, which results in potentiation of transactivation of the ER (Kato et al. 1995, Bunone et al. 1996, Tremblay et al. 1999). In contrast, estrogen has been shown to downregulate MAPK activation in vascular SMC (Morey et al. 1997). Furthermore, in rabbit cardiac allografts and aortic SMC, estradiol-treatment inhibits IGF-1 mRNA and protein expression (Lou et al. 1997, Lou et al. 1998b). Estrogens also downregulate PDGF-A mRNA in monocytes and vascular SMC *in vitro* (Shanker et al. 1995, Kikuchi et al. 2000).

There has been incomplete information on at which level estrogen intervenes in the growth factor – MAPK cascade. In this study, estradiol-treatment downregulated the injury-induced expression of IGF-1, PDGF-Rα, and PDGF-B mRNA and protein in the male rat aorta, and with immunohistochemistry, an inhibitory effect on PDGF-A protein expression was observed as well (IV). Decreased amounts of growth factors, or their receptors, are likely to result in decreased activation of downstream proteins, such as MAPK, c-myc, and c-fos (Morey et al. 1997), and decreased proliferation and migration of SMC in the vessel wall.

The most prominent changes were seen in the expression of IGF-1, which is a rather weak mitogen alone (Ferns et al. 1991a). However, IGF-1 is an important progression factor for PDGF-stimulated proliferation (Banskota et al. 1989, DeAngelis et al. 1995), and furthermore, changes in IGF-1/IGF-1R interactions modulate the effects of VEGF in the vasculature (Smith et al. 1999, Hellstrom et al. 2001). The understanding of these interactions adds special significance to the regulation of IGF-1 expression by estrogen.

PDGF-A has been thought to exert mainly mitogenic effects (Majesky et al. 1990, Rekhter and Gordon 1994). Increased expression of PDGF-A, and -Rα have also been reported in rat cardiac allograft arteriosclerosis and obliterative bronchiolitis (Kallio et al. 1999, Sihvola et al. 1999), and shown to correlate with increased allograft arteriosclerosis (Sihvola et al. 1999). This suggests that the inhibition of the PDGF/PDGF–Rα axis by estrogen may inhibit the mitogenic responces in the developing neointima, and furthermore, the development of, not only restenosis, but also of allograft arteriosclerosis.

The variability in the PDGF-A and PDGF-B expression in Western blotting and immunohistochemistry was likely due to the growth factors' predominant localization in vascular neointima. Neointima comprised of only a minor part of the vessel, whereas most of the protein for Western blotting originated from the media and adventitia. Changes in media and adventitia, as quantitated by immunohistochemistry, were small or non-existent, and this was probably reflected in Western blotting as rather small changes in growth factor protein expression.

Finally, it seems logical that the predominant receptor in the cell/tissue determines the estrogenic response to growth factors. In the uterus, where ERα predominates, estrogen-treament results in cell proliferation, as a concequence of increased production of growth

factors, which in turn may strengthen the ERα-mediated transactivation. Also in vascular ECs, estrogen induces proliferation through ERα, which results in increased reendothelialization after vascular injury (Krasinski et al. 1997, Somjen et al. 1998, Brouchet et al. 2001). In contrast, in the vascular SMC, where ERβ is the dominant subtype (I, II), estrogen-treatment is accompanied by decreased growth factor production (IV), resulting in downregulation of cell proliferation and cell migration. Indeed, it could be speculated that ERα and ERβ have a kind of "ying-yang" relationship in the body, with ERα inducing cell growth and ERβ repressing it. In addition, ERβ may act as a dominant negative regulator of ERα-mediated signalling (Pettersson et al. 2000, Lindberg et al. 2003). In the uterus, ERβ has been reported to modulate the uterotrophic responses induced by ERα, by reducing IGF-1 synthesis and the resulting cell proliferation (Weihua et al. 2000).

4. Is there a role for estrogen in regulating the influx of precursor cells?

Although both replication and migration contribute to neointimal thickening, it has been suggested that migration may be more important in the process (Clowes and Schwartz 1985, Du Toit et al. 2001). Furthermore, as mentioned, most of the cells contributing to the thickened neointima seem to originate either from circulating or bone marrow-derived vascular precursors, or both (Hillebrands et al. 2000, Saiura et al. 2001, Shimizu et al. 2001, Hu et al. 2002, Sata et al. 2002, Tanaka et al. 2003). Their route to the vessel wall is not clear, although some studies suggest that adventitia has an important role in neointima formation since adventitial activation precedes neointima formation (Shi et al. 1996a, Shi et al. 1996b, Frosen et al. 2001). Also, growth factors, such as PDGF and VEGF, have been proposed to be important regulators of the differentiation of vascular precursor cells, either to SMCs or ECs, respectively (Yamashita et al. 2000). Estrogen has been shown to attenuate adventitial fibroblast migration *in vitro* (Li et al. 1999) as well as adventitial activation after rat carotid balloon injury *in vivo* (Oparil et al. 1999). Furthermore, estrogen significantly enhanced endothelial progenitor cell production, mobilization, and incorporation in the recovering artery in response to vascular injury. As estrogen significantly inhibited the outgrowth, proliferation, and migration of cells from aortic explants (III), and better than the proliferation of cultured vascular SMC, it would be interesting to hypothesize that estrogen inhibits the proliferation of neointimal (precursor) cells at extravascular sites, such as bone marrow, which results in reduced influx of precursor cells into the vascular wall. This needs further investigation.

5. Current controversies on postmenopausal hormone replacement therapy

In recent years, the pros and cons of hormone replacement therapy have come under intense scrutiny. Large randomised prospective trials, such as the Heart and Estrogen/Progestin Replacement Study (HERS) and Women's Health Initiative (WHI), reported that treatment of postmenopausal women with oral conjugated equine estrogen (CEE) plus medroxyprogesterone acetate (MPA) did not have any overall benefits on cardiovascular disease, and furthermore, might increase the risk for breast cancer (Hulley et al. 1998, Rossouw et al. 2002). The women participating in the HERS had pre-existing coronary heart disease, and although the WHI was described as a primary prevention trial, a remarkable portion of the participants had risk factors for CHD (smoking, obesity, diabetes, hypercholesterolemia), and/or received antihypertensive medication, statins, or aspirin, and some even had a history of cardiovascular events and/or interventions. Also, only one third of the women started the therapy close to their menopause – the average patient age was 63 years. Thus, current data indicate that estrogens are not beneficial in the secondary prevention of CHD, whereas the data on their efficacy in primary prevention is strong (Stampfer et al. 1985, Stampfer et al. 1991, Grady et al. 1992, Grodstein et al. 1996, Grodstein et al. 2000). Observational studies, such as the Nurses' Health study, on the other hand, have been critisized on the grounds that their results might reflect the "healthy woman effect", i.e. women who take estrogens tend to be more educated, have more favourable lifestyles, fewer risk factors, and are more compliant with treatment than women, who do not take the estrogens. However, it is obvious that randomized double-blind primary prevention studies in perimenopausal women (with menopausal symptoms) are extremely difficult, if not impossible, because the relieve of menopausal symptoms would clearly unmask the treatment and control groups.

Supporting evidence on estrogen's benefits in primary, but not secondary, prevention of CHD comes from studies in monkeys: primary prevention resulted in 70% decrease in coronary artery atherosclerosis, whereas HRT initiated at 2 years after the menopause was ineffective (Williams et al. 1995, Adams et al. 1997, Clarkson et al. 1998, Clarkson et al. 2001). The failure to show any cardiovascular benefits in the randomized clinical trials has also been claimed to be due to several other factors besides the timing of the treatment, such as the type of estrogen used (CEE vs. estradiol), the route of administration (oral vs. transdermal), or the impact of progestin. *In vitro*, estrone, estriol and estrone sulfate, which constitute a major part

of CEE, have little or no inhibitory effects on vascular SMC proliferation, migration, and MAP kinase activity, whereas estradiol is effective (Dubey et al. 2000). It has also been reported that transdermal estrogen may reduce serum triglycerides and leave CRP levels unchanged, contrary to oral treatment (Crook et al. 1992, Strandberg et al. 2003). The data on whether progestins, and particularly MPA, interfere with estrogen's vascular effects are controversial: some claim that progesterone is detrimental whereas others find no effect (Adams et al. 1990, Williams et al. 1994, Grodstein et al. 1996, Levine et al. 1996, Adams et al. 1997, Miyagawa et al. 1997, Gerhard et al. 1998, Grodstein et al. 2000, Kawano et al. 2001). It has been suggested that other progestins, such as norethisterone, are more favourable than MPA on estrogen's vasculoprotective functions and may even enhance them (Alexandersen et al. 1998, Ylikorkala et al. 2000).

This thesis supports the clinical evidence on estrogen's benefits in the primary prevention of vasculoproliferative disorders. Estradiol treatment right after surgical menopause significantly inhibited neointimal thickening in the rat. Moreover, the vasculoprotective effects of estrogen, or SERMs, were sustained when the treatment was discontinued on day 14, i.e. no rebound effect was observed. This suggests that the critical time interval in the development of intimal hyperplasia is before day 14, and supports the benefit of early treatment (III). Indeed, in the injured rat vessel, the protective responses occur rather early after injury: proliferation peaks at 3 days in the media, at 4 days in the adventitia, and at 7 days in the neointima; and the responses gradually subside to a low level after 14 days post-injury (Du Toit et al. 2001, Frosen et al. 2001). Also, our more recent preliminary findings suggest that the vascular precursor cell influx peaks at 2-4 days post-injury and falls to a low level after 14 days (Luoto NM et al., unpublished results). Although the impact of delayed treatment on neointimal hyperplasia was not studied here, our findings may explain why the studies on the role of estrogen in the secondary prevention of cardiovascular disease failed; the treatment was initiated too late.

6. Estrogen receptors as targets for vasculoselective drug therapy

Attempts to target single genes in order to prevent vasculoproliferative disorders are usually not sufficient because other pathways remain active. Estrogens are tempting candidates for vasculoselective drug therapy as they are capable of regulating multiple genes at the same time. As the risks and advantages of HRT are debated, the development of tissue-selective

estrogens has become of interest. The potential alternatives today are SERMs, ERα/ERβ-selective ligands, and phytoestrogens. An ideal compound would have beneficial effects on menopausal symptoms, cardiovascular system, bone, and brain, while being neutral in its effects on uterus and the breast, and carrying no risk for venous thromboembolism (Table 5). The absence of gender-related side-effects might make it applicable to men. Unfortunately, no such compounds are known today.

Tamoxifen and raloxifene have favourable effects on plasma lipids, breast and bone but they do not alleviate menopausal symptoms. Ospemifene has no adverse effects on post-menopausal symptoms, is neutral in its effects on hot flashes, and furthermore, has an estrogenic effect on vagina (Voipio et al. 2002, Rutanen et al. 2003). Previous studies have mainly concentrated on the effects of SERMs on lipid-induced changes in the arteries. This study shows the potential of the clinically used (tamoxifen and raloxifene) and two novel (ospemifene and fispemifene) SERMs to preserve estrogenic effects on the vascular injury response in the rat, i.e. the inhibition of SMC proliferation and migration as well as the enhancement of reendothelialization (III). It was notable that at least in the rat, the vasculoprotective doses were higher than the clinically used ones, which suggests that the current clinical dosing is incapable of achieving maximal vasculoprotection. Also the *in vitro* doses were over the physiological estradiol serum levels – however, serum estradiol concentrations may not be equal to tissue concentrations, since local estrogen synthesis may play a role also in the vasculature (Murakami et al. 2001).

ERβ-selective compounds have now become available (Meyers et al. 2001, Harris et al. 2003). Their effects on the vascular wall are not yet known. Current data strongly suggest that ERβ is an important regulator of SMC biology after vascular injury and does not induce uterine growth (I, II), which makes ERβ-selective ligands potential candidates for vasculo-selective drug design. Furthermore, the predominant expression of ERβ in the inflammatory cells of the cardiac allografts (II), and the anti-inflammatory action of ERβ-selective ligands (Harris et al. 2003), as well as the finding that ERβ-/- mice develop a myeloproliferative disease resembling human chronic myeloid leukemia with lymphoid blast crisis (Shim et al. 2003), suggest that ERβ-selective ligands might also have a potential therapeutic role in the prevention of immune activation after transplantation, and the subsequent development of transplant arteriosclerosis,

i.e. both as immunoregulatory and as vasculoprotective therapy. This merits further investigation.

Table 5. Comparison between the effects of HRT, SERMs, and ER-selective ligands in different tissues

	Vascular SMC	Vascular EC	Lipids	Bone	Breast (ca)	Uterus	Vagina	Hot flushes	References
HRT	-	+	-	+	+	+	+	-	MacGregor and Jordan 1998, Morello et al. 2002
Tamoxifen	-	+	-	+	-	+	-	+	MacGregor and Jordan 1998, Morello et al. 2002; III
Raloxifene	-	+	-	+	-	0	-	+	MacGregor and Jordan 1998, Morello et al. 2002; III
Ospemifene	-	+	-	+	-	0	+	0	Morello et al. 2002; III
Fispemifene	-	+	-	+	-	0	?	?	Lauri Kangas, personal communication; III
ERα-agonist	0?	+?	-	+*	+*	+*	?	-*	*Harris et al. 2002
ERβ- agonist	-?	0?	?	0*	0*	0*	?	?	*Harris et al. 2003
Ideal SERM	-	+	-	+	-	0	+	-	

CONCLUSIONS AND SUMMARY

The development of a hyperplastic neointima in restenosis and in allograft arteriosclerosis is a protective response to mechanical, immunological, or infectious injury to the vascular wall. The pathogenesis of these disorders resembles, in many respects, those of common atherosclerosis, including inflammation, influx of proliferating SMC to the injury site, extracellular matrix synthesis, and finally, vessel remodelling (Fig. 11). Furthermore, the current data suggest that neointimal SMC-like cells derive mainly, not from the vascular media, but from circulating precursor cells. Estrogen's beneficial effects in the primary prevention of cardiovascular disease are well-established. However, estrogen therapy alone has some important side-effects, such as the growth-promoting effects in the uterus and breast. The data in this thesis support estrogen's role in vasculoprotection; particularly in preventing the proliferation and migration of SMC to the injured artery, and the subsequent neointima formation. Estrogen receptor β is the predominant receptor in the vascular wall after mechanical and immunological injury, colocalizing with the vascular SMC in the media and neointima, while ERα remains at a low level only (Fig. 11). ERβ also predominates in the arteries, veins, myocardium, and inflammatory cells of cardiac allografts, but shows no correlation to the intensity of rejection. Furthermore, this study shows for the first time, that the vasculoprotective effects of estrogen can be differentiated from its uterotrophic effects with specific targeting to ERβ. The findings make ERβ a potential candidate for estrogen-based vasculoselective drug design. SERMs bind both ERs with variable affinities, and have a mixed estrogen agonist/antagonist profile in different tissues. This study shows that some SERMs have beneficial estrogen agonist effects on the developing neointima after balloon injury, while being neutral or weakly estrogenic in the uterus. In addition, the study supports the hypothesis that early intervention of the injury-response is of paramount importance, and in some cases even sufficient to repress the response. Finally, estrogen interacts with growth factor signalling cascades, also in the vascular wall. It simultaneously inhibits the expression of IGF-1, PDGF, and PDGF-Rα mRNA and protein, and the concomitant proliferation and migration of vascular SMC, which is possibly one mechanism behind estrogen's vasculoprotective properties (Fig. 11).

INJURY

ALLOANTIGEN
INDEPENDENT

INFECTION

ALLOANTIGEN
DEPENDENT

INFLAMMATORY
RESPONSE

precursor cell

macrophage B cell T cell thrombocyte

cytokines

adhesion molecules
MHC class II
ET-1

EC

NOS
prostacyclin synthase

IGF-1
PDGF

SMC

ERβ

ERβ

growth factors
ECM synthesis

MIGRATION

PROLIFERATION

REMODELLING
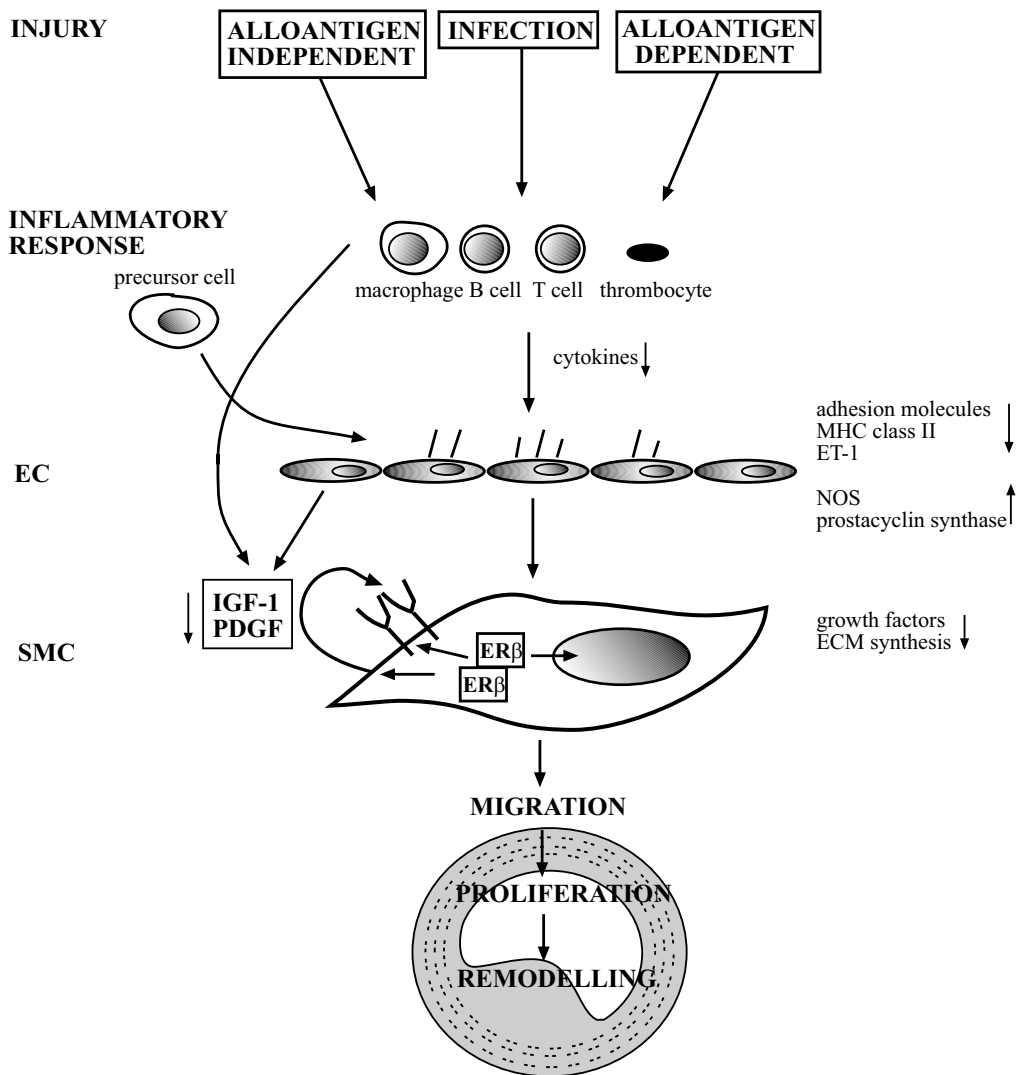
Figure 11. *Potential targets of estrogen action in the development of vascular intimal hyperplasia. Whether the endothelial injury is mechanical, infectious, or alloimmune in origin, it initially leads to an inflammatory response. The injured endothelium expresses numerous proinflammatory molecules and adhesion molecules, which mediate the entry of the inflammatory cells to the vessel wall. The activated inflammatory cells replicate and secrete cytokines, which, in turn, stimulate endothelial cells (EC) and vascular smooth muscle cells (SMC). SMC migration and proliferation within the arterial intima is induced by growth factors and vasoactive peptides released from the activated leukocytes, and SMC themselves, which leads to persistent paracrine and autocrine proliferation. Vascular precursor cells are thought to contribute significantly to the development of neointimal hyperplasia. Estrogen therapy downregulates adhesion molecules, several proinflammatory cytokines, major histocompatibility (MHC) antigens, growth factors, and extracellular matrix (ECM) synthesis in the vascular wall. In addition, it increases the activity of nitric oxide synthase (NOS) and prostacyclin synthase, which results in vasodilation. Finally, estrogen receptors interact with growth factor signalling cascades. Estrogen receptor (ER) β is the predominant estrogen receptor in vascular SMC, and thus may primarily mediate the vasculoprotective actions of estrogen. ET-1; endothelin.*

YHTEENVETO (FINNISH SUMMARY)

Valtimon sisimmän kerroksen, intiman, paksuuntuminen ja siitä johtuva suonen umpeutuminen on tyypillinen muutos valtimonkovettumataudissa (ateroskleroosi) sekä sepelvaltimoiden pallolaajennuksen jälkeisessä uudelleenahtautumisessa (restenoosi). Immunosuppressiivisen lääkityksen kehittyminen on parantanut elinsiirtojen lyhytaikaisennustetta. Krooninen rejektio, jonka keskeinen manifestaatio on siirteen valtimoiden arterioskleroosi, on kuitenkin edelleen tärkein syy elinsiirteiden menetykseen. Lisäksi noin puolet siirteistä menetetään toimivina, pääasiassa kardiovaskulaaritapahtumien vuoksi.

Edellä mainittujen tautien patofysiologialle on ominaista verisuonen sisäpintaa suojaavan endoteelikerroksen vaurio, joka johtaa verisuonen seinämän tulehdusreaktioon, sileälihassolujen kerääntymiseen vauriokohtaan sekä niiden jakautumiseen (proliferaatio) ja vaeltamiseen (migraatio) suonen seinämässä, valtimon seinämän paksuuntumiseen ja uudelleenmuotoutumiseen. Tärkeässä asemassa ovat erilaiset kasvutekijät ja sytokiinit, joita mm. verisuonen endoteelisolut, sileälihassolut ja tulehdussolut erittävät. Viimeaikaiset tutkimukset viittaavat lisäksi siihen, että paksuuntuneen neointiman muodostavat sileälihassolun kaltaiset solut ovat peräisin verenkierron kantasoluista, eivät verisuonen keskikerroksesta, mediasta, kuten aikaisemmin uskottiin.

Estrogeeni on steroidihormoni, joka säätelee solujen jakautumista ja erilaistumista elimistön eri osissa. Estrogeenin verisuonia suojeleva vaikutus on osoitettu laajalti sepelvaltimotaudin ehkäisyssä ja tutkimukset eri eläinmalleissa ja soluviljelmissä tukevat vahvasti tätä havaintoa, vaikka viimeaikaisten seurantatutkimusten tulokset ovatkin olleet päinvastaisia. Huomattava estrogeenin sivuvaikutus on kuitenkin sen vaikutus kohtuun: estrogeenihoito lisää kohdun limakalvon syöpäriskiä ja voi nopeuttaa rintasyövän etenemistä. Aikaisemmin oli vaikea ymmärtää, miten estrogeenihoito voi samanaikaisesti stimuloida solujen jakautumista joissakin kudoksissa ja estää sitä toisaalta. Toisen estrogeenireseptorin (ER), ERβ:n, löytyminen 'klassisen' ERα:n rinnalle lisäsikin merkittävästi ymmärrystämme estrogeenin vaikutusmekanismeista eri kudoksissa.

Tämän väitöskirjatyön tavoitteena oli tutkia eri estrogeenireseptoreiden ilmenemistä pallolaajennuksen tai siirteen hyljintäreaktion aiheuttamassa verisuonen umpeutumisessa sekä

selvittää mahdollisia mekanismeja estrogeenin verisuonivaikutusten taustalla, keskittyen erityisesti verisuonen sileälihassolujen säätelyyn. Tavoitteena oli myös estää valtimon seinämän paksuuntumista estrogeenireseptoreihin eri tavoin sitoutuvilla aineilla. Työssä käytettiin rotan kaulavaltimon ja aortan pallolaajennusmalleja sekä rotan ja ihmisen sydänsiirtonäytteitä. Sileälihassolu- ja kudosviljelmissä tutkittiin estrogeenien vaikutusta solujen migraatioon ja proliferaatioon.

Tämän tutkimuksen tulokset tukevat teoriaa, jonka mukaan estrogeenilla on merkittäviä verisuonia suojaavia ominaisuuksia: estrogeeni estää annosriippuvaisesti verisuonen sileälihassolujen proliferaatiota ja migraatiota sekä paksuuntuneen intiman muodostumista (I, III, IV). Tutkimus osoittaa, että ERβ on vallitseva reseptori valtimon seinämän sileälihassoluissa verisuonivaurion jälkeen, kun taas ERα ilmenee ainoastaan matalalla tasolla (I). ERβ ilmenee voimakkaasti myös sydänsiirteen valtimoissa, laskimoissa, sydänlihaksessa ja tulehdussoluissa (II). Estrogeenin hyödylliset verisuonivaikutukset voidaan lisäksi erottaa sen haitallisista kohtua kasvattavista vaikutuksista aineilla, jotka sitoutuvat voimakkaasti ERβ:aan (I). Nämä havainnot tekevät ERβ:sta potentiaalisen verisuoniselektiivisen lääkesuunnittelun kohteen.

Selektiiviset estrogeenireseptorin säätelijät (SERM) sitoutuvat molempiin estrogeenireseptoreihin eri voimakkuuksilla ja vaikuttavat estrogeenin tavoin tietyissä kudoksissa ja antiestrogeenin tavoin toisissa kudoksissa. Tämä työ osoittaa, että osa SERM:sta estää pallolaajennuksen jälkeistä restenoosia estrogeenin tavoin stimuloimatta kohdun kasvua (III). Verisuonivaurion aiheuttamien vasteiden esto välittömästi vaurion jälkeen on erityisen tärkeää, kun taas pitkään jatkuvalla hoidolla on vähemmän merkitystä.

Väitöskirja osoittaa lisäksi, että estrogeenihoito vähentää eri kasvutekijöiden ilmenemistä verisuonen seinämässä mekaanisen vaurion (pallolaajennus) jälkeen (IV). Se estää insuliinin kaltaisen kasvutekijän (IGF-1), verihiutaleperäisen kasvutekijän (PDGF) sekä PDGF-reseptori α:n muodostumista verisuonessa, mikä on mahdollisesti yksi tärkeä mekanismi estrogeenin hyödyllisten verisuonivaikutusten taustalla.

SAMMANDRAG (SWEDISH SUMMARY)

Förtjockning av artärens innersta lager, intiman, och därav förorsakad förträngning av blodkärlet är en typisk förändring vid blodkärlsförkalkning (ateroskleros) och återförträngning av kransarterärna efter ballong-dilatation (restenos). Framsteg i utvecklingen av immunosuppressiv medicinering har förbättrat transplantationers korttidsprognos. Kronisk rejektion, vars centrala manifestation är arterioskleros i det transplanterade organets artärer, är dock fortfarande den viktigaste orsaken till att transplantat trots allt förloras. Kring hälften av transplantaten förloras därtill funktionsdugliga, främst på grund av kardiovaskulära händelser.

Typiskt för ovan nämnda sjukdomars patofysiologi är en skada på endotellagret som skyddar blodkärlens insida. Skadan orsakar en inflammationsreaktion i blodkärlsväggen samt en ansamling av glatta muskelceller vid skadan och deras proliferation och migration inom blodkärlsväggen, och slutligen, en förtjockning och omformning av artärväggen. Olika tillväxtfaktorer och cytokiner, vilka utsöndras av bland annat blodkärlens endotelceller, glatta muskelceller och inflammationsceller, spelar en viktig roll i processen. Därtill har nyligen publicerade undersökningar påvisat, att de glatta muskelcellsliknande celler som bygger upp den förtjockade neointiman kunde härstamma från stamceller i blodcirkulationen och inte från blodkärlets mellanlager, median, så som man tidigare trott.

Estrogenet är ett steroidhormon som reglerar celldelning och -differentiering i olika delar av kroppen. Estrogenets blodkärlsskyddande effect har påvisats vitt i primarprevention av ateroskleros, och undersökningar i olika djurmodeller och cellexperiment stöder fynden, trots att det på senaste tid publicerats rapporter med motsatta resultat. Estrogenets betydande bieffekt är dess effekt på livmodern: estrogenterapin ökar risken för cancer i livmoderslemhinnan och kan försnabba progressionen av bröstcancer. Tidigare var det svårt att förstå hur estrogenterapi samtidigt kan stimulera celldelning i vissa vävnader och samtidigt hindra den på andra håll. Dock ökade upptäckten av den andra estrogenreceptorn (ER)β, vid sidan om den 'klassiska' ERα, avsevärt vår insikt i estrogenets verkningsmekanismer i olika vävnader.

Målet med detta avhandlingsarbete var att undersöka förekomsten av olika estrogenreceptorer i blodkärlsförtjockning efter ballongdilatation eller transplantationsrejektion, samt att utreda möjliga mekanismer bakom estrogenets blodkärlsverkan, med betoning på regleringen av

blodkärlens glatta muskelceller. Målet var också att hindra artärväggens förtjockning med ämnen som binder sig olika till de två estrogenreceptorerna. I arbetet användes ballongdilatationsmodeller på råttors halsartär och aorta, samt prover från råttors och människors hjärttransplantat. Estrogenernas verkan på cellmigration och -proliferation undersöktes med cell- och vävnadsodlingar.

Undersökningsresultaten i denna avhandling stöder teorin enligt vilken estrogenet har betydande blodkärlsskyddande egenskaper: estrogenet hämmar dosrelaterat proliferation och migration av blodkärlens glatta muskelceller, samt en förtjockning av intiman. (I, III, IV). Undersökningarna påvisar även att ERβ är den dominerande receptorn i artärväggens glatta muskelceller efter blodkärlsskada, medan ERα endast förekommer i låga nivåer (I). Förekomsten av ERβ är stor även i artärer, vener, hjärtmuskel och inflammationsceller i hjärttransplantat (II). Estrogenets nyttiga blodkärlseffekter kan dessutom åtskiljas från dess skadliga effekter på livmodern med ämnen som binder kraftigt vid ERβ (I). Dessa fynd gör ERβ till ett potientiellt mål för blodkärlsselektiv läkemedelsdesign.

Selektiva estrogenreceptormodulatorer (SERM) binder till båda estrogenreceptorer med olika affinitet och verkar estrogenlikt i vissa vävnader och antiestrogenlikt i andra vävnader. Det här arbete påvisar, att en del av SERM hämmar restenos efter ballongdilatation liksom estrogen, utan att stimulera tillväxten av livmodern (III). Viktigt är att kunna hämma blodkärlsskadans effekter omedelbart efter skadan, långtidsterapihar en mindre betydelse.

Avhandlingen påvisar även att estrogenbehandling minskar förekomsten av olika tillväxtfaktorer i blodkärlsväggen efter mekanisk skada vid ballongdilatation (IV). Estrogenbehandlingen hindrar bildandet av insulinlik tillväxtfaktor 1 (IGF-1), trombocytderiverad tillväxtfaktor (PDGF) och PDGF-reseptor α i blodkärlen, vilket kan vara en viktig mekanism bakom estrogenets blodkärlsskyddande effekt.

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