Studies on the regulation of plasminogen activation-dependent processes by retinoids and steroid hormones

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STELLINGEN

1. Estrogenen induceren de expressie van de mannose receptor in de lever.
(dit proefschrift)

2. Endotheel bezit een actief, cytochroom P450 gemedieerd, retinoïnezuur metaboliserend vermogen *(dit proefschrift)* en levert hiermee een belangrijke bijdrage aan het retinoïnezuur metabolisme in vivo.

3. De retinoïnezuur receptor β-gemedieerde inductie van t-PA synthese door retinoïnezuur in gekweekte endotheelcellen is een voorbeeld van receptor subtype specificiteit.
(dit proefschrift)

4. Het vermogen om 9-*cis* retinoïnezuur te vormen via isomerisatie van all-*trans* retinoïnezuur is, in tegenstelling tot in hepatocyten, niet aanwezig in endotheelcellen.
(dit proefschrift)

5. De verlaagde t-PA plasma concentraties bij het gebruik van orale contraceptiva of hormoon substitutie therapie kunnen verklaard worden door een stimulatie van de klaring van t-PA door estrogenen.
(dit proefschrift)

6. Gezien de hoge prevalentie van cellulaire immuniteit tegen het adenovirus in de mens, lijkt het gebruik van de huidige vectoren gebaseerd op adenovirus type 5, niet geschikt voor arteriële gentherapie.
(Schulick et al., J Clin Invest 99:209-219, 1997)

7. De resultaten van Ashcroft et al. leveren geen direct bewijs dat estrogenen wondheling versnellen via een toename in TGF-β1 secretie door huid fibroblasten.
(Ashcroft et al., Nat Med 3:1209-1215, 1997)

8. *In vitro* studies naar transcriptie regulatie die geen rekening houden met de chromatinestructuur verwaarlozen een belangrijk aspect van transcriptieregulatie.

9. In de studie van Pepper et al., waarin het effect van reeds bekende angiogenese remmers op *in vitro* angiogenese in collageengelen wordt vergeleken met hun effect op de proteolytische activiteit van de endotheelcellen, zou het bestuderen van collagenase activiteit een logischere keuze zijn geweest dan bestudering van PA activiteit.
(Pepper et al., J Cell Bioch 55: 419-434, 1994)

10. Het is onbegrijpelijk dat in films en op de televisie geweld steeds meer tot amusement wordt verheven, terwijl in het dagelijks leven de verontwaardiging over de toepassing van geweld toeneemt.

11. Gezien het grote papierverbruik tijdens een promotieonderzoek zou iedere promovendus na zijn promotie een boom moeten planten.

12. Voeding is meer dan de naam doet vermoeden.

Stellingen behorende bij het proefschrift: *" Studies on the regulation of plasminogen activation-dependent processes by retinoids and steroid hormones".*

Leiden, 4 juni 1998 Mirian Lansink

There are more things in heaven and earth,
than are dreamt of in your philosophy.

Shakespeare (1564-1616)
from: Hamlet, I.V.

Aan mijn ouders

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CHAPTER 1

GENERAL INTRODUCTION

CONTENTS

1.1 INTRODUCTION

The plasminogen/plasmin system is a highly regulated enzymatic cascade for obtaining controlled extracellular proteolysis in many biological processes involving matrix degradation, tissue remodeling and destruction, and cell migration. Processes as diverse as proteolytic degradation of fibrin clots (fibrinolysis), reproduction, embryonic development, wound healing, angiogenesis, tumour cell invasion, atherosclerosis and brain function are dependent on the plasminogen activation system. Given the extraordinary number of physiological and pathophysiological contexts in which the plasminogen activation system has a role, the activity of the system is highly regulated and sensitive to a variety of cytokines, growth factors, hormones and vasoactive compounds to ensure that its activity is confined in space and time.

In this thesis the effects of hormones on two physiological processes in which the plasminogen activation system is very important, viz. intravascular fibrinolysis and angiogenesis, have been studied. In particular, the molecular mechanisms involved in the hormonal regulation of the synthesis and clearance of activation factors have been investigated. The following paragraphs will briefly introduce the plasminogen activation system, intravascular fibrinolysis, and the angiogenic process. Subsequently, reported effects of hormones on intravascular fibrinolysis and angiogenesis will be summarized and current ideas on how these hormones could exert their action are described. Finally, the outline and aims of the thesis are given.

1.2 THE PLASMINOGEN /PLASMIN SYSTEM

Plasminogen, present in high concentrations in blood and other body fluids, constitutes a reservoir of potential proteolytic activity. There are two types of plasminogen activators, the tissue-type (t-PA) and the urokinase-type (u-PA). Both are capable of catalyzing the conversion of the inactive zymogen plasminogen to the active serine proteinase plasmin, which can degrade fibrin as well as extracellular matrix proteins. A simplified scheme of the plasminogen/plasmin system is shown in Figure 1. It is generally thought that the primary role of t-PA is to generate plasmin for blood fibrinolysis, while u-PA is associated mostly with tissue proteolysis. However, recent experiments in mice with targeted disruption of the genes for t-PA and u-PA (so-called t-PA and u-PA "knock-out" mice) suggest that these distinct roles of t-PA and u-PA may be less strict than assumed (Carmeliet et al., 1994, 1995).

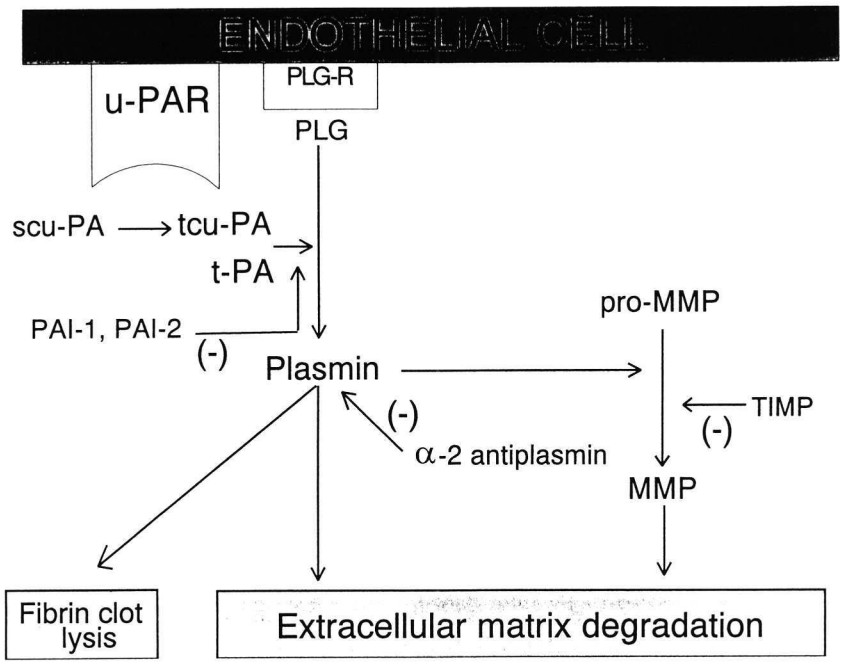

Figure 1. The plasminogen activation system. Abbreviations used are: t-PA, tissue-type plasminogen activator; u-PA, urokinase plasminogen activator; scu-PA, single chain u-PA; tcu-PA, two-chain u-PA; u-PAR, u-PA receptor; PAI, plasminogen activator inhibitor; PLG, plasminogen; PLG-R, plasminogen receptor (PLG-R); MMP, matrix- metallo-proteinase; TIMP, tissue-inhibitor of matrix-metallo-proteinases.

t-PA differs from other proteins involved in haemostasis in that it is secreted from cells as an active enzyme. The form of u-PA initially released from cells is the single-chain zymogen form pro-uPA, scu-PA, with an activity that is at least several hundred-fold lower than that of two-chain u-PA (tcu-PA). Conversion of scu-PA to tcu-PA can be catalyzed by plasmin.

There are two inhibitors of t-PA and u-PA, of which PAI-1 is usually the physiologically relevant one. The physiological importance of PAI-2 remains unclear (Dear and Medcalf, 1995). Plasmin is inhibited by α_2-anti-plasmin.

1.3 INTRAVASCULAR FIBRINOLYSIS

1.3.1 General

The blood coagulation and the fibrinolysis (i.e. plasminogen /plasmin) systems determine the balance between the formation and dissolution of blood clots, but also contribute to the pathogenesis of cardiovascular diseases such as atherosclerosis, thrombosis and restenosis

(Collen and Lijnen, 1991). Insufficient lysis of fibrin, the matrix structure of thrombi and hemostatic plugs, may result in an interruption of the normal blood flow, whereas excessive, premature fibrin degradation can lead to bleeding (Lijnen and Collen, 1989). As a consequence, fibrinolysis needs to be finely regulated in time, location and extent.

Results from *in vitro* studies indicate that t-PA is the key enzyme in the onset of intravascular fibrin degradation (Wun and Capuano, 1985, 1987), whereas u-PA may be more involved in later stages of fibrin dissolution (Gurevich, 1988). The activity of t-PA in the circulation is regulated at several levels:

(i) *Secretion from endothelial cells.* It is generally assumed that plasma t-PA mainly originates from the vascular endothelium (Kooistra et al., 1994). Vascular endothelial cells are ideally positioned to contribute to plasma levels of t-PA and contain, *in vivo,* large amounts of t-PA, as shown by histochemical, immunohistochemical and biochemical techniques. t-PA can be secreted from endothelial cells in two ways, the so-called constitutive secretion route and the regulated secretion route ("acute release"). During constitutive secretion, the synthesized t-PA is exported continuously, ensuring a basal plasma level of t-PA. In the other way, t-PA is concentrated and stored in specialized storage vesicles. The contents of these storage pools are only delivered to the extracellular compartment in response to specific extracellular stimuli. Both pathways are subjected to regulation by diverse compounds (reviewed by Kooistra et al., 1996a).

(ii) *Rapid hepatic clearance.* t-PA is rapidly cleared from the circulation via receptor-mediated endocytosis. This clearance is mainly mediated via two independent receptor systems in the liver, the mannose receptor on liver endothelial cells and Kuppfer cells (Kuiper et al., 1988; Smedsrød and Einarsson, 1992; Otter et al., 1990) which recognizes mannose-rich oligosaccharides as present on t-PA, and the low-density-lipoprotein receptor related protein (LRP) on liver parenchymal cells (Bu et al., 1992). The LRP has also been shown to be a receptor for t-PA complexed with PAI-1 (Orth et al., 1994). Blockade of the mannose receptor or the LRP in mice has been shown to prolong the plasma half-life of radiolabelled human recombinant t-PA from less than 1 minute to 3-4 and 4-5 minutes, respectively, whereas simultaneous inhibition of both receptor systems increased plasma half-life to more than 20 minutes (Narita et al., 1995). Similar results were found in rats, where blockade of the mannose receptor or the LRP decreased t-PA clearance by 60%, whereas blockade of both receptors lowered t-PA clearance by 90% (Biesen et al., 1997).

(iii) *Interaction with PAI-1.* PAI-1 is considered to be the most relevant inhibitor of t-PA under physiological conditions (Sprengers and Kluft, 1987). Consistent with its important role in controlling intravascular fibrinolysis, high plasma PAI-1 levels are correlated with an increased risk of recurrent myocardial infarction (Hamsten et al., 1987) and with deep venous thrombosis (Juhan-Vague et al., 1987; Nguyen et al., 1988), whereas genetic deficiencies in PAI-1 have been associated with bleeding disorders (Schleef et al., 1989; Fay et al., 1992;

Kruithof et al., 1995; Fay et al., 1997). Although the origin of plasma PAI-1 is not known with certainty, hepatocytes, adipocytes, platelets and endothelial cells are usually suggested to contribute to synthesis and plasma presence of PAI-1 (Erickson et al., 1984; Booth et al., 1985; Loskutoff, 1991; Chomiki et al., 1994; Thornton and Gelehrter, 1995; Shimomura et al., 1996).

(iv) *Activity is confined to fibrin.* In the absence of fibrin, t-PA is a poor enzyme, but fibrin strikingly enhances the activation rate of plasminogen by t-PA by at least two orders of magnitude. Besides t-PA, plasminogen is also able to bind to a fibrin clot via its so-called lysine binding sites, and so a ternary complex is formed. Fibrin essentially increases the local plasminogen concentration by creating an additional interaction between t-PA and its substrate. The high affinity of t-PA for plasminogen in the presence of fibrin thus allows efficient activation on the fibrin clot, while no efficient plasminogen activation by t-PA occurs in plasma (Collen and Lijnen, 1991). Additionally, plasmin bound to fibrin is only slowly inactivated by α_2-antiplasmin, whereas free plasmin, when formed, is rapidly inhibited by α_2-antiplasmin (Wiman and Collen, 1978).

Importantly, t-PA is much more effective in degrading fibrin if added before clot formation than after clot formation has occurred (Brommer, 1984; Zamaron et al., 1984; Fox et al., 1985). This difference in potency can be several hundred-fold and explains at least in part the large therapeutic doses of t-PA required in thrombolytic therapy. It also emphasizes the preventive potential of a proper endogenous fibrinolytic capacity to counteract the development of intravascular thrombosis.

1.3.2 Effect of hormones on plasma fibrinolytic capacity

Several hormones have been shown to modulate the intravascular fibrinolytic capacity by modulating plasma t-PA and PAI-1 levels.

Estrogens. In man, high estrogen status has been found to be associated with lowered t-PA and PAI-1 antigen levels in several different situations. (1) Premenopausal women have significant lower plasma levels of t-PA and PAI-1 than men of comparable age, whereas in postmenopausal women these levels are similar to those observed in men (Gebara et al., 1995). (2) Several prospective studies show that women using oral contraceptives containing 20-37.5 µg ethinylestradiol and 75-750 µg progestogens have decreased t-PA antigen levels (Gevers Leuven et al., 1987; Petersen et al., 1993; Winkler et al., 1996) and PAI-1 antigen levels (Petersen et al., 1993; Quehenberger et al., 1993; Winkler et al., 1996). (3) A number of studies have demonstrated that hormone replacement therapy with orally administered estrogens with or without the concomitant administration of progestogens lower plasma t-PA and PAI-1 levels in postmenopausal women (Kroon et al., 1994; Gebara et al., 1995; Gilabert et al., 1995; Winkler et al., 1995; Shahar et al., 1996; Koh et al., 1997). (4) In male-to-female transsexual subjects, exogenous ethinylestradiol in combination with a testosterone

antagonist, cyproterone acetate, significantly reduced plasma levels of t-PA and PAI-1, whereas in female-to-male subject receiving testosterone the plasma levels of t-PA and PAI-1 were not significantly changed (Kooistra et al., 1996b).

Progestogens. A few studies investigated the effect of progestogen-only preparations on plasma fibrinolytic activity. Continuous long-term treatment with levonorgestrel as a depot appeared to reduce fibrinolytic activity (Singh et al., 1992a, 1992b). Orally administered low or high doses of progestogens, or short-term depot progestogens did not alter plasma fibrinolytic activity (reviewed in Kuhl, 1996). Interpretation of these studies on progestogens is hampered, however, by the fact that fibrinolytic activity rather than t-PA and PAI-1 plasma concentration was measured. Several studies have reported a modulating effect of progestogens on estrogen-induced changes in fibrinolysis factors, with the effect being dependent on the kind of progestogen used (Sabra and Bonnar, 1983; Gevers Leuven et al., 1987).

Androgens. Androgens have been shown to be inversely correlated with plasma PAI-1 activity in several studies (Caron et al., 1989; Yang et al., 1993; Glueck et al., 1993; Phillips et al., 1994; Anderson et al., 1995). With the exception of a study with hyperlipidemic men, in which a positive relationship between circulating plasma t-PA levels and endogenous testosterone levels was demonstrated (Glueck et al., 1993), no correlation between plasma testosterone and t-PA levels has been found (Anderson et al., 1995).

Retinoids. Retinoids, i.e. natural and synthetic analogues of vitamin A, effectively and rather specifically stimulate t-PA synthesis in cultured human endothelial cells without markedly altering PAI-1 production (Kooistra et al., 1991; Thompson et al., 1991; Bulens et al., 1992). *In vivo* studies in rats show that plasma t-PA levels and retinoid status are correlated: t-PA activity in vitamin A-deficient rats is about three times lower than control values, while supplementation of the diet of normal rats with retinoic acid (vitamin A acid) increases plasma t-PA activity up to about 50% above control values (Kooistra et al., 1991; Bennekum et al., 1993). In contrast to t-PA, plasma PAI-1 levels in rats are not correlated with retinoid status (Bennekum et al., 1993). These *in vivo* data illustrate the physiological relevance of retinoids in determining plasma t-PA levels, but also indicate that under normal physiological conditions retinoid levels in plasma and tissues may be sufficient to maintain almost maximal retinoid-dependent t-PA synthesis *in vivo*. This may also explain why treatment of humans with isotretinoin (the 13-*cis* isomeric form of retinoic acid) did not alter (Wallnöfer et al., 1993) or only marginally increased ($\sim$ 1.3-fold) plasma t-PA levels (DeClerck et al., 1993). More extensive testing of retinoids in humans is hampered by the fact that its use is associated with side effects (Biesalski, 1989).

1.4 ANGIOGENESIS

1.4.1 General

Angiogenesis is a multistep process leading to the formation of new capillaries by "sprouting" from preexisting blood vessels. This process involves degradation of the vascular basal membrane and the fibrin or interstitial matrix by endothelial cells; migration and proliferation of endothelial cells; and finally the formation of new capillary tubes and a new basement membrane (Folkman, 1986). Angiogenic activity is generally low in the adult organism, except for the normal cycling changes that occur in the female reproductive tract (Findlay, 1986). Only during injury and certain pathological conditions do other tissues exhibit angiogenesis, for example in rheumatoid arthritis (Lahita, 1985), diabetic retinopathy (Engerman, 1989), wound healing (Arnold and West, 1991) and solid tumour growth (Folkman, 1990).

In vitro models, in which the formation of capillary structures of endothelial cells in three-dimensional matrices of fibrin and collagen has been studied (Pepper and Montesano, 1990; Koolwijk et al., 1996), have pointed to the role of matrix degrading proteolytic enzymes (Pepper et al., 1996), and among the most relevant proteolytic systems involved herein are the plasminogen activator system, especially u-PA and the metalloproteinases (MMPs) (Bacharach et al., 1992; Van Hinsbergh, 1992; Colville-Nash and Seed, 1993). It has been demonstrated that the formation of capillary-like tubular structures in three-dimensional fibrin matrices is suppressed by interfering with u-PA activity or the binding of u-PA to the u-PA receptor (Pepper and Montesano, 1990; Koolwijk et al., 1996).

It is generally thought that u-PA has a role in the regulation of the first steps of angiogenesis. Upon secretion the inactive scu-PA binds to its cell-surface receptor, u-PAR, and is converted to its proteolytic active form (Blasi et al., 1994). Receptor-bound u-PA converts receptor-bound plasminogen into plasmin, which degrades fibrin and various other matrix glycoproteins, and activates several MMPs, notably interstitial collagenase (MMP-1), stromelysin-1 (MMP-3) and gelatinase B (MMP-9) (Nagase, 1994). In the cell environment, active u-PA is immediately inhibited by PAI-1, thereby keeping the action of u-PA localized to the cell surface (Loskutoff, 1991) and preserving matrix integrity and normal tissue architecture (Pepper et al., 1996). The binding of u-PA to the u-PAR concentrates the u-PA activity in foci at the endothelial cell surface (Blasi et al., 1994; Danø et al., 1994).

In addition to generating plasmin, the u-PA:u-PAR complex is also required for cell attachment and motility: proteolysis of cell-matrix interactions will not cause invasion of a cell into a matrix unless new cell-matrix connections are simultaneously formed (Dejana, 1996). The cell has to pull itself into the matrix by alternating attachment and detachment of cell-matrix interactions.

Several factors have been identified which modulate angiogenesis. Among the most

extensively studied angiogenic inducers are fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) which regulate endothelial cell proliferation, migration and extracellular proteolytic activity (Ferrara et al., 1992; Dvorak et al., 1995; Pepper et al., 1996). In many angiogenic situations inflammatory mediators such as tumour necrosis factor alpha (TNFα) and transforming growth factor alpha (TGFα) are also present (Klagsbrun and D'Amore, 1991), and evidence is accumulating that angiogenesis and chronic inflammation are codependent (Jackson et al., 1997). In several settings, such as wound healing, inflammation, and tumour growth, fibrin is a major component of the extracellular matrix in which angiogenesis occurs. In contrast to matrices composed of collagen or other macromolecules, fibrin constitutes a provisional matrix that is progressively remodelled and replaced by other matrix components, including collagen (Pepper, 1997).

1.4.2 Effects of hormones and retinoids on angiogenesis

An association between angiogenic activity and steroid hormones was initially suggested by experiments in which endometrial tissue was transplanted to the anterior chamber of the monkey eye (Marker, 1940) and to the hamster cheek pouch (Abel, 1985). In both models, the extent of vascularization and incidence of bleeding were influenced by ovarian steroids. These experiments support the concept that the angiogenic response in the endometrium is, at some level, dependent on the presence of estrogen and progesterone (Iruela-Arispe et al., 1996).

The use of different model systems, the parameter being measured, the concentration of steroid used, and species differences have complicated comparison and interpretation of different studies. For example, many studies on angiogenesis have used the behaviour (for example proliferation or migration) of cultured endothelial cells as an index of angiogenic response. However, such measures are limited in that they tend to focus on individual components of the angiogenic process rather than the complete response of capillary tube formation. The use of such assays to study the effect of steroids on angiogenesis has produced somewhat conflicting results, which will be discussed in the next paragraphs.

Estrogens. Low concentrations of estradiol (1-10 nM) increased migratory and proliferative activity of human umbilical vein endothelial cells (Morales et al., 1995). Estradiol also induced proliferation of bovine endothelial cells with a reduced stimulation at high concentrations (Brandi et al., 1993). Estradiol had no effect on angiogenesis in the rabbit cornea (Gross et al., 1981; Yamamoto et al., 1994), but produced avascular zones in the chorio-allantoic membrane (CAM) assay at low concentrations (Gagliardi and Collins, 1993) and inhibited the outgrowth of capillary-like microvessels from rat aorta explants embedded in collagen gels (Jaggers et al., 1996). On the other hand, in mice ovariectomy markedly

decreased *in vivo* vascularisation of Matrigel plugs containing basic FGF (bFGF); estrogen replacement increased angiogenesis to the levels observed in non-ovariectomized mice (Morales et al., 1995).

Progesterone. Like estrogens, progesterone effects vary, depending on the assay system used. Progesterone (10 μg/ml) produced degeneration of microvessels, which were grown out of rat aorta explants embedded in collagen gels, but only after 7 days of treatment (Jaggers et al., 1996). In the rabbit corneal system in the presence of bFGF, progesterone did not affect angiogenesis (Yamamoto et al., 1994), whereas its metabolite medroxyprogesterone acetate, inhibited angiogenesis in this system (Jikihara et al., 1992; Yamamoto et al., 1994).

Androgens. Variable effects were also reported for androgens. In the rabbit corneal assay, testosterone had no effect, whereas 5α-dihydrotestosterone was inhibiting angiogenesis (Gross et al., 1981; Yamamoto et al., 1994). Androstenedione inhibited microvessel growth from a rat aorta explant after 11 days (Jaggers et al., 1996). In contrast to these inhibitory effects of androgens on angiogenesis, androgen deprivation in men caused suppression of prostate cancer angiogenesis (Marshall and Narayan, 1993; Jospeh and Isaacs, 1997).

Thyroid hormones. No effects of thyroid hormones on angiogenesis have been described until now.

Dihydroxyvitamin D3. Studies on the effects of vitamin D on angiogenesis have been limited. The active compound of vitamin D, 1,25-dihydroxyvitamin D_3, inhibited the migration of human pulmonary endothelial cells and inhibited angiogenesis in the CAM assay (Oikawa et al., 1990; Hisa et al., 1996).

Glucocorticoids. Steroids such as cortisol and tetrahydro-S-(11-deoxytetrahydrocortisol) have been termed "angiostatic steroids" because of their inhibitory activity in the CAM assay (Crum et al., 1985). Several studies have also shown an inhibitory effect of dexamethasone, a synthetic glucocorticoid, on angiogenesis in diverse model systems like sponge- and colon-cancer induced angiogenesis in rats (Hu et al., 1994), bFGF-induced angiogenesis in the rabbit corneal assay (Yamamoto et al., 1994), microvascular invasion of rabbit growth plate cartilage (Brown et al., 1990) and microvessel density in brain tumours in rats (Wolff et al., 1993).

Retinoids. In most assay systems retinoids have been shown to inhibit angiogenesis. Several retinoids, including all-*trans* retinoic acid, retinyl acetate and the synthetic retinoid CH55, inhibit embryonic neovascularization, as tested in the CAM assay (Ingber and Folkman, 1988; Oikawa et al., 1989). Also, tumour-induced angiogenesis is inhibited by retinoids; systemic treatment of mice with all-*trans*, 9-*cis* and 13-*cis* retinoic acid inhibited cutaneous angiogenesis induced by tumour cells injected intradermally in the skin (Majewski et al., 1994). Induction of angiogenesis by tumour cell-conditioned media in the rat cornea is also inhibited by all-*trans* retinoic acid (Lingen et al., 1996). Furthermore all-*trans* retinoic acid hinders the capacity of bovine microvascular endothelial cells to invade collagen gels and to

form tubules (Pepper et al., 1994). All-*trans*-N-(4-hydroxyphenyl) retinamide (4HPR or fenretinide), a synthetic retinoid, is also able to inhibit angiogenesis in the CAM assay and to inhibit the formation of tubules in an *in vitro* model with bovine pulmonary artery endothelial cells (Pienta et al., 1993). Its mechanism of action is not known, and it had even been assumed that it is not a true retinoid because of the absence of a terminal carboxyl group. Fanjul et al. (1996) recently observed, however, that fenretinide activates only a selected portion of the broad retinoid receptor response, and this might be the basis for its specific biological activities. Reported effects of retinoids on individual components of the angiogenic process are less uniform. For instance retinol (vitamin A), all-*trans* and 9-*cis* retinoic acid have been shown to augment proliferation of cultured human endothelial cells, whereas in another study all-*trans* retinoic acid (modestly) inhibited proliferation of large and small vessel endothelial cells (Spencer Green, 1994; Lingen et al., 1996). In aggregate, these studies on the effects of hormones on angiogenesis show potent (modulating) activities of these compounds, but apparently lack consistency as a result of differences in assay systems.

1.5 NUCLEAR HORMONE RECEPTORS

1.5.1 Introduction

Unlike the water-soluble peptide hormones and growth factors, which bind to cell surface receptors, lipophilic hormones such as steroids, retinoids, thyroid hormones and vitamin D_3 can pass through the cell membrane and bind to specific (nuclear) receptors. The nuclear receptors function to control the activity of target genes directly (for review see Ribeiro et al., 1995; Mangelsdorf et al., 1995; Beato et al., 1996).

Nuclear receptors primarily act through direct association with specific DNA sequences known as hormone response elements (HREs). The HREs are composed of six base-pair half-site sequences that are organized as palindromic or direct repeats. The nuclear receptors bind mainly as dimers to the response element, and each monomer interacts with a half-site sequence within the response element. Receptor-binding specificity is determined by the primary nucleotide sequence, but also by the orientation and spacing between the two half-sites of the response element (Umesono et al., 1991; Mangelsdorf et al., 1995). After binding to DNA, the receptor is thought to interact with components of the basal transcriptional machinery and with sequence-specific transcription factors (Horwitz et al., 1996).

Because of the structure/function and sequence relationships, the term superfamily is used to encompass all of the known nuclear hormone receptors. The superfamily is often further divided into the steroid receptor family, the thyroid/retinoid/vitamin D_3 (or nonsteroid) receptor family, and the orphan receptors, a group of nuclear receptors that have been cloned but where the putative activating ligands remain to be identified. Each type of nuclear receptor constitutes a subfamily (for example, the retinoic acid receptor [RAR] subfamily).

Receptor subtypes are the products of individual genes (for example, RARα, RARβ, RARγ), and receptor isoforms are the products of alternate splicing, promoter usage, or both (for example, RARα1, RARα2).

The nuclear receptors share a common structural and functional organization with distinct domains (Fig 2A). The amino-terminal domains (A/B) vary markedly in size among family members (Evans, 1988). This domain contains transactivation functions which seem to be hormone-independent and may interact with other transcription activation proteins in complex response elements (Hollenberg and Evans, 1988; Tora et al., 1989; Bocquel et al., 1989; Ribeiro et al., 1995). The central DNA-binding C-domain is highly conserved and contains two zinc-finger motifs for DNA interaction. It confers specificity for binding to HREs and is also involved in receptor dimerization (Freedman and Luisi, 1993; Glass, 1994). The ligand-binding domain in the carboxyterminal region (E/F) is also relatively well-conserved. This domain confers specificity for ligand binding and is involved in receptor dimerization, transcription activation and nuclear translocation (Picard and Yamamoto, 1987; Green and Chambon, 1988; Hollenberg and Evans, 1988; Tora et al., 1989; Fawell et al., 1990; Glass, 1994)

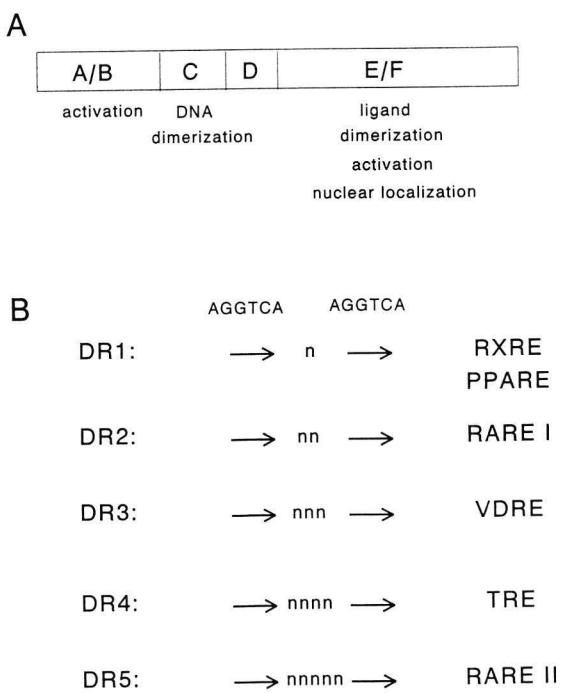

Figure 2. (A) Schematic representation of the functional domains of nuclear receptors. The structure of nuclear receptors can be divided into six domains, A, B, C, D, E and F. The function of the different domains is indicated below the domains.
(B) The "1 to 5" rule. Direct repeats (DR) with a variable number of intervening nucleotides (n) serve as hormone response elements for the retinoid X receptor (RXRE), peroxisome-proliferator-activated receptor (PPARE), thyroid hormone receptor (TRE), vitamin D_3 receptor (VDRE) and the retinoic acid receptor (RARE). The RXRE and PPARE are direct repeats with 1 intervening nucleotide (DR1). The RARE is a DR2 or a DR5. A DR3 serves as a VDRE and a DR4 as a TRE. The concensus sequence of the direct repeats is indicated.

1.5.2 The steroid receptor family

This family consists of receptors for glucocorticoids (GR), mineralocorticoids (MR), progesterone (PR), androgens (AR) and estrogens (ER). Unliganded steroid receptors are bound in a large multiprotein complex of chaperones, including heat-shock proteins (hsps), which maintains the receptors in an inactive but ligand-friendly conformation (Pratt, 1993). Upon hormone binding, the receptor: hsp complexes dissociate and the activated receptors bind, exclusively as homodimers, to palindromic DNA sequences, separated by 3 nucleotides. GR, MR, PR and AR recognize the same consensus sequence, AGA ACA nnn TGT TCT (Klock et al., 1987), while ER recognizes the consensus sequence, AGG TCA nnn TGA CCT (Martinez et al., 1987), identical with the half-sites used by the nonsteroid nuclear receptors (see below). The similarity in HREs for the various receptors would create a specificity problem, if transcriptional activation by hormones is merely dependent on the binding of a (dimeric) receptor complex to a response element. It is becoming clear now, that transcriptional regulation by receptors is controlled by many other factors, including promoter architecture, receptor phosphorylation, and recruiting of corepressors and coactivators in response to hormone (see Perlmann and Evans, 1997 for a recent review).

Besides their ability to stimulate gene activity, nuclear hormone receptors are also competent transcriptional repressors (Beato et al., 1995). Transcriptional repression can occur by competition for the DNA binding site, by competition for common mediators to the transcription initiation complex, and by sequestration of transcription factors such as AP-1 and NF-κB into inactive forms.

1.5.3 Thyroid hormone, retinoic acid and vitamin D_3 receptors

Among the nonsteroid nuclear hormone receptors are the retinoic acid receptor (RAR), the retinoid X receptor (RXR), the vitamin D_3 receptor (VDR) and the thyroid hormone receptor (TR). In contrast to the steroid receptors, these receptors all appear to be tightly bound to the nucleus at specific sites in the DNA in the absence of ligand, and they frequently silence basal promoter activity (Tsai and O'Malley, 1994). Each of the receptors can recognize the common consensus half-site sequence, AGG TCA, that can be configured into a variety of structured motifs, usually in the form of a direct repeat (Umesono et al., 1991). In contrast to the steroid receptors, which function as homodimers, high-affinity binding of RAR, VDR and TR to their cognate HREs requires heterodimer formation with RXR.

The specificity of the hormonal response is achieved by, among others, the property of each receptor to recognize HREs that are unique in their spacing of the direct repeats (DRs). Binding and transactivation assays using direct repeats with variable spacer lengths led to the postulation of the "3-4-5" rule: a direct repeat of AGG TCA separated by 3 nucleotides (DR-3) is a vitamin D_3 response element (VDRE); by 4 nucleotides (DR-4) a TRE; and by 5 nucleotides (DR-5) an RARE (Näär et al., 1991; Umesono et al., 1991). This model was

subsequently extended to the "1-to-5" rule to reflect the fact that a DR-1 serves as an RXR response element and a DR-2 is a second RARE (reviewed by Mangelsdorf et al., 1994).
The "1-to-5" rule is depictured schematically in Fig. 2B. The "1-to-5" rule suffers from numerous exceptions when confronted with the wide variety of the response elements identified in "natural" retinoic acid, thyroid hormone and vitamin D_3 responsive genes (see Pfahl et al., 1994), underlining that the mechanism of hormone action is still far from being understood.

In addition to spacing, subtle differences in the sequence of the hexad half-site and the 5' extension of these response elements are also important and can have strong effects on the activity of a receptor (Vivanco Ruiz et al., 1991; Mader et al., 1993; Willy et al., 1995).

1.5.4 Presence of response elements for nuclear hormone receptors in t-PA and u-PA promoter Bulens et al. (1995) identified a functional RARE at -7319 base pairs (bp) in the t-PA promoter, consisting of a DR5 of the GGG TCA motif. Subsequently, the same group showed the presence of a multihormone responsive enhancer located between -7.1 and -9.5 kbp (see Table 1 for details), which confers responsiveness to glucocorticoids, progesterone and androgens, but not estrogens (Bulens et al., 1997). Nonetheless, a t-PA promotor/chloramphenicol acetyltransferase reporter construct, containing 7144 bp upstream sequence of the t-PA promoter, was found to be responsive to estradiol. A computer search for an ERE in the t-PA gene revealed a sequence between position -1392 to -1380 differing only in two nucleotides from the consensus ERE (Kooistra et al., 1990).

For the u-PA promoter, no functional response elements for steroid hormones or retinoids have been described so far.

1.6 SCOPE OF THE STUDY

From the above it is evident that the regulation of plasminogen activation-dependent processes is influenced by steroid hormones and retinoids, as exemplified by their effects on intravascular fibrinolysis and angiogenesis. It also became clear that different hormones may exert different effects. However, the exact molecular mechanism(s) via which the hormones exert their actions as well as the parameter(s) being influenced in the different plasminogen activation systems remained often unknown, and are the subject of this study.

First, we will concentrate on the role of the various retinoid receptor subtypes and retinoid metabolism in the retinoid-stimulated t-PA expression in cultured human endothelial cells. Secondly, we will deal with the lowering of plasma t-PA levels in the presence of estrogens. Because estrogens do not affect t-PA synthesis in cultured human endothelial cells (Kooistra et al., 1990, 1996), we will test the hypothesis that circulating t-PA levels are decreased by estrogens via increased hepatic clearance. Thirdly, we have investigated the molecular

SITE	SEQUENCE	COORD (bp)	MOTIF
RARE			
t-PA/DR0	TCACCT TGAACT	-9 431	DR0/inv
t-PA/DR4	TGACCC attg TGAACC	-8 682	DR4/inv
t-PA/DR2	AGGTGG tg TCACCT g AGGTCA gg AGTTCG	-8 396	IR2-ER1-DR2
t-PA/ER8	TGATCT cattgccg AGGTGA	-7 402	ER8
t-PA/DR5	**GGGTCACCC tg GGGTCA**	**-7 319**	**DR5-ER2**
GRE			
t-PA/GRE	AGAACT	-7 474	GRE half site
t-PA/GRE	AGAACG	-7 428	GRE half site
t-PA/GRE	**AGCACA tgc AGTCTC**	**-7 501**	**PAL3/inv**
t-PA/GRE	**TGTACA gtt AGAGTG**	**-7 703**	**PAL3/inv**
t-PA/GRE	**AGGGCT ctg TGTTCT**	**-7 942**	**PAL3**
t-PA/GRE	**GGTCCA cag TGTTCA**	**-7 960**	**PAL3**

Table 1. Putative and functional retinoic acid receptor and glucocorticoid receptor response elements in the human t-PA promoter (according to Bulens 95, 97). The functional response elements are indicated in bold capitals. Recognition motifs are identified as direct (DR), everted (ER), inverted (IR) and (partial) palindromic (PAL) repeats with the number of intervening nucleotides indicated. Coordinates (Coord) of elements located in the human t-PA gene are numbered relative to the start site of transcription.

mechanisms by which hormones modulate tube formation in an *in vitro* angiogenesis model. Many studies on angiogenesis with cultured endothelial cells are limited in that they tend to focus primarily on individual components of the angiogenic process (matrix degradation, cell proliferation or migration) rather than (also) on the complete response of capillary-like tube formation. We have used a human *in vitro* model in which human microvascular endothelial cells can be induced to invade a three-dimensional fibrin matrix thereby forming capillary-like tubular structures (Koolwijk et al., 1996). This model mimicks the *in vivo* situation where fibrin appears to be a common component of the matrix at sites of chronic inflammation, and in which neovascularization proceeds rapidly if adequate angiogenic stimuli are present (Dvorak et al., 1995).

1.7 OUTLINE AND AIMS OF THE THESIS

The aim of this thesis was to study the effects of hormones in two situations in which the plasminogen activation system is very important, intravascular fibrinolysis and angiogenesis. In particular we will focus on the molecular mechanism by which retinoids stimulate t-PA synthesis in cultured human endothelial cells, the role of retinoid metabolism in the retinoid-stimulated expression of t-PA, the effects of estrogens on the clearance of t-PA by the liver, and the effects of, and molecular mechanisms by which, steroid hormones and retinoids affect the formation of capillary-like tubular structures in an *in vitro* angiogenesis model.

In Chapters 2 and 3, the molecular mechanism involved in the retinoid-stimulated t-PA synthesis in cultured human endothelial cells was studied. As a first step to determine which retinoic acid receptor sybtype(s) are primarily involved in the t-PA induction, we characterized the expression and (retinoid) regulation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in cultured human umbilical vein endothelial cells (HUVEC) by Northern-blot analysis (Chapter 2). Furthermore, by the use of receptor-subtype specific agonists and antagonists the receptor subtype involved in the t-PA induction by retinoids was studied. The studies in Chapter 3 were directed at elucidating the subsequent steps in this retinoid-induced t-PA synthesis in HUVEC by using antisense oligonucleotides directed against receptor subtype specific isoforms.

In Chapter 4 studies were performed to determine whether a rapid metabolism of retinoic acid by HUVEC *in vitro* could explain the rather high concentrations (1-10 μM) which are necessary to induce optimally t-PA synthesis *in vitro* compared to *in vivo,* where plasma retinoic acid levels (1-10 nM) apparently suffice to maintain nearly maximal t-PA expression. To this purpose the metabolism of all-*trans* retinoic acid by HUVEC and, for comparison, by hepatocytes, a recognized site for retinoic acid metabolism, was investigated. Studies on the uptake and metabolism of 9-*cis* retinoic acid in cultured human endothelial cells and in hepatocytes were also performed.

Plasma levels of proteins are the resultant of their synthesis and their clearance. The studies in Chapter 5 were conducted to determine whether the decreased t-PA plasma antigen levels associated with high estrogen status are related to changes in the clearance of t-PA. *In vivo* studies in rats and mice were performed in which plasma clearance of endogenous and exogenous t-PA was determined. The role of the two main receptor systems for t-PA clearance, the mannose receptor system and the LDL-receptor related protein system (LRP), was assessed by injection of specific competitors of these receptors and by adenoviral vector-mediated overexpression of receptor associated protein (RAP).

In Chapter 6 the effects of hormones on angiogenesis and u-PA synthesis were investigated. For these studies an *in vitro* angiogenesis model was used in which human microvascular endothelial cells are grown on top of a three-dimensional fibrin matrix and are

stimulated to form capillary-like tubular structures by the continuous presence of basic fibroblast growth factor and tumour necrosis factor α.

In Chapter 7 the general conclusions that can be drawn from this work are discussed.

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CHAPTER 2

INVOLVEMENT OF RETINOIC ACID RECEPTOR α IN THE STIMULATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR GENE EXPRESSION IN HUMAN ENDOTHELIAL CELLS

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SUMMARY

Retinoids stimulate tissue-type plasminogen activator (t-PA) gene expression in human endothelial cells, and are likely to do so by binding to one or more nuclear retinoid receptors. The present study was initiated to identify the retinoid receptor(s) involved in this process. Expression and regulation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) were analyzed by Northern-blot analysis of total or poly(A)-rich RNA prepared from cultured human umbilical vein endothelial cells (HUVEC). Prior to any exposure to retinoids, HUVEC express two transcripts for RAR-α (3.6 kb and 2.8 kb), and low levels of transcripts for RAR-β (3.4 kb and 3.2 kb) and RAR-γ (3.3 kb and 3.1 kb). Two RXR subtypes were identified, RXR-α (4.8 kb) and, at a much lower concentration, RXR-β (2.4 kb); no evidence for the presence of RXR-γ was found. Furthermore, HUVEC express cellular retinol-binding protein I (CRBP-I) and cellular retinoic-acid-binding protein I (CRABP-I) mRNA. Exposure of HUVEC to 1 μM retinoic acid or the retinobenzoic acid, Ch55, led to the induction of the two RAR-β mRNAs, RXR-α mRNA and CRBP-1 mRNA, whereas the expression of the other receptor and CRABP-1 transcripts did not change appreciably. Using retinoid analogues that bind preferentially to one of the RAR or RXR subtypes, we found evidence that RAR-α is involved in the retinoid-induced t-PA expression in HUVEC. This conclusion was strengthened by experiments in which blocking of RAR-α with a specific RAR-α antagonist, Ro 41-5253, was demonstrated to suppress the induction of t-PA by retinoids.

INTRODUCTION

Impairment of the endogenous fibrinolytic system has been implicated in the development of thromboembolic disease [1]. Consequently, compounds that can enhance the fibrinolytic capacity of the blood would provide a potential therapeutic and preventive approach to thrombosis. Vascular endothelial cells represent a direct target for increasing the fibrinolytic capacity of the blood since they synthesize and release both tissue-type plasminogen activator (t-PA), a key enzyme in the onset of the fibrinolytic process, and a specific t-PA inhibitor, plasminogen-activator inhibitor type 1 (PAI-1) [2]. This has led to an interest into the mechanisms involved in the regulation of t-PA and PAI-1 expression in endothelial cells. A variety of compounds have been shown to stimulate t-PA synthesis in human endothelial cells, including the vasoactive agents thrombin and histamine [3-5], short-chain fatty acids such as butyrate [6], protein kinase C activators such as phorbol 12-myristate 13-acetate [7-9], triazolobenzodiazepines [10], and retinoids (i.e. natural and synthetic analogues of vitamin A) [11-13]. Among the physiologically relevant compounds, retinoids have also been shown to enhance t-PA levels in plasma and tissues *in vivo* without significant elevation of PAI-1 [11,14], and thus seem attractive candidates for stimulating endogenous fibrinolysis. Their potential future usefulness will depend, however, on

the availability of appropriate synthetic derivatives which are more potent than retinoic acid (which gave maximally about 50% increase of rat plasma t-PA levels [11,14] and which have less side effects, including a reduced teratogenic risk [15]. Recent discoveries now furnish possibilities of designing more effective retinoids and dissociating increased efficacy from side effects. Firstly, using cultured human umbilical vein endothelial cells (HUVEC) as a model, we and others have identified synthetic retinoids which are more potent than retinoic acid in stimulating t-PA expression and which can be used as lead compounds for further development [11,13]. Secondly, the understanding of the molecular mechanism by which retinoid signals can be transduced at the transcriptional level has been greatly advanced by the discovery of two classes of nuclear retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) [16]. These receptors are members of the steroid-receptor superfamily of proteins that function as dimeric, ligand-dependent transcription factors [16]. They modulate transcription through binding to specific retinoic-acid- response elements of their target genes [16]. Three RARs, RAR-α, -β, and -γ, encoded by distinct genes, have been identified [17-22]. From each RAR gene, several isoforms can be derived by alternative splicing and alternative promoter usage [23-25]. Also, three distinct RXRs, RXR-α, -β, and -γ, have been described [26-29]. Specific spatio-temporal patterns of expression for each RAR gene [30-34] and RXR gene [29], as shown by *in situ* hybridization studies on embryo sections, as well as the striking interspecies amino acid sequence conservation of a given retinoid receptor type or isoform, which is much higher than the similarity between either the three RAR or the three RXR types within a given species, suggest that each RAR type (and isoform) and RXR type may specifically control the expression of subsets of retinoid target genes [25,35]. This multitude of targets may allow separation of toxic liabilities and, therefore, enhance the therapeutic benefit of retinoids by use of receptor-subtype-selective ligands [36].

We hypothesize that RARs/RXRs may be involved in mediating the action of retinoids on t-PA induction in HUVEC. As a first step in testing this hypothesis, we have characterized the RAR and RXR transcripts in HUVEC and investigated the retinoid receptor subtype involved in the induction of t-PA gene expression by use of receptor-subtype-selective ligands and a receptor- subtype-specific antagonist.

MATERIALS AND METHODS

Materials

All-*trans* retinoic acid (retinoic acid) was purchased from Sigma Chemical Co. The retinobenzoic acids Am80, Am580 and Ch55 [37,38] were generously provided by Drs. Y. Hashimoto and K. Shudo, Faculty of Pharmaceutical Sciences, University of Tokyo, Japan. 9-*cis* retinoic acid (Ro 4-4079) and the RAR-α selective antagonist, Ro 41-5253 [39], were kindly provided by Drs. C. Apfel and M. Klaus, Hoffmann-LaRoche Ltd., Basle, Switzerland. The structures of the retinoids, the retinobenzoic acids, and the RAR-α antagonist used in the experiments are shown (Fig. 1). Stock solutions of the retinoids, retinobenzoic acids, and the RAR-α antagonist were prepared in Me_2SO and preserved at -20°C for no longer than 14 days. Immediately before use, the various compounds were diluted in incubation medium to final test concentrations. The final concentration of Me_2SO did not exceed 0.1% (by vol.). To eliminate light, all experiments were performed in subdued light as much as possible and the tubes containing the retinoids were covered with aluminium foil.

Enzyme immunoassay kits for determination of human t-PA antigen (Thrombonostika t-PA) were obtained from Organon Teknika and for determination of human PAI-1 antigen ("Imulyse") were obtained from Biopool. Deoxycytidine 5[α-^{32}P]-triphosphate was from Amersham International plc. Other materials used in the methods described have been specified in detail in the relating references.

Cell culture experiments

Endothelial cells were isolated from human umbilical cord veins using collagenase by a technique similar to that described by Jaffe et al. [40]. Cells were grown in as described [42] at 37°C in a 5% CO_2 atmosphere in fibronectin-coated dishes in Dulbecco's modified Eagles medium supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) human serum, 10% (by vol.) newborn calf serum (heat-inactivated), 2 mM L-glutamine, 5 U/ml heparin, 150 µg/ml endothelial cell growth supplement [41] and penicillin/streptomycin. The medium was replaced every 2-3 days. Subcultures were obtained by trypsin/EDTA treatment at a ratio of 1:3.

For experiments, confluent cultures were used at the second or third passage, and cells were always refed with incubation medium the day before the experiment, viz. Dulbecco's modified Eagles medium supplemented with 20 mM Hepes, 10% (by vol.) human serum (containing < 1 ng/ml of retinoic acid), 2 mM L-glutamine and penicillin/streptomycin. Conditioned media were obtained by incubating cells at 37°C for 24, 48, or 72 h, with incubation medium containing the appropriate concentration of the test compound or stock solvent (final concentration maximally 0.1%, by vol). The media were refreshed every 24 h. Conditioned media were centrifuged for 2 min in a Beckman Microfuge to remove cells and cellular debris, and samples were frozen at -20°C until use. Cells were washed at 37°C three times with 0.15 M NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, and were used for isolation of RNA.

RNA hybridization

Total RNA was extracted from endothelial cells as described by Chomczynski and Sacchi [43]. RNA samples were dissolved in H_2O. The RNA concentration in each sample was determined spectrophotometrically. Poly(A)-rich RNA was selected by oligo(dT)-cellulose chromatography. Equal amounts of RNA from the different dishes were analyzed for their CR(A)BP, RAR and RXR subtypes, and t-PA, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase mRNA contents by Northern blot hybridization. With Northern blotting, RNA samples were subjected to gel electrophoresis in formaldehyde/agarose gels, as outlined by Maniatis et al. [44], then transferred to Hybond N according to the instructions of the manufacturer. Prehybridization and hybridization were at 61°C (1 mM EDTA, 7% SDS, 0.5 M NaH_2PO_4/Na_2HPO_4, pH 7.2; modified from [45]). DNA fragments to be used as probes were isolated from low- melting agarose [44]. Hybridization was usually performed with 1 ng/ml probe labelled by random prime labelling to approximately 2 x 10^8 cpm/µg DNA [44].

The filters were washed at a stringency of 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0, containing 1% SDS for2 x 15 min at 61°C [44]. The membranes were subsequently exposed to an Amersham Hyperfilm-MP film with an intensifying screen at -80°C. For quantification of the relative amounts of mRNA, densitometry was used. In short, a scan of the bands was made on a CS 910 Shimadzu scanner and the areas under the peaks were integrated and plotted with the aid of a United Technology Packard data processor.

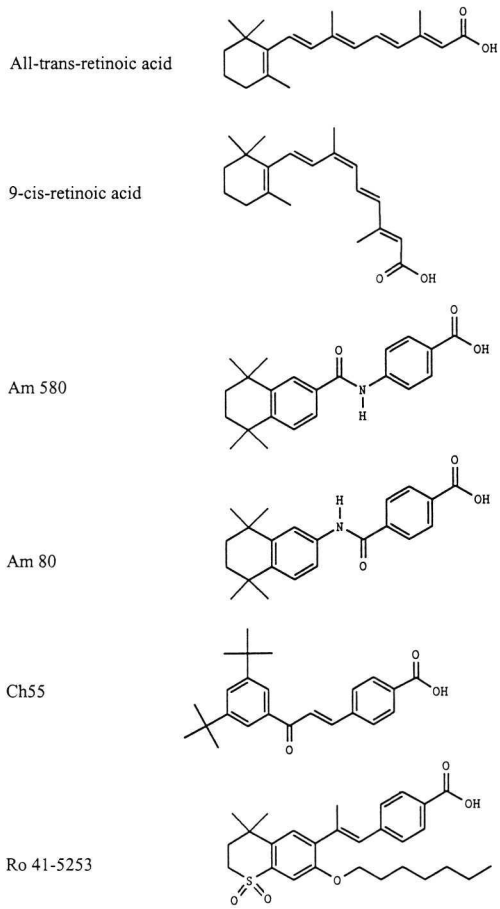

Figure 1. Structure of retinoids, retinobenzoic acids and RAR-α antagonist.

cDNA probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.3-kb *Eco*RI-*Bgl*II fragment of a human RAR-α cDNA, a 1.4-kb *Bam*HI-*Xba*I fragment of a human RAR-β cDNA, a 1.4-kb *Eco*RI-*Xba*I fragment of a mouse RAR-γ1 cDNA, in which a few bases have been changed in the A and F regions by site-directed mutagenesis so that it encodes the human amino acid sequence; a 1.4-kb *Eco*RI-*Bgl* II fragment of a human RXR-α cDNA, and a 1.4-kb *Bam*HI-*Eco*RI fragment of human RXR-γ. The RAR-α and RXR-α were treated by PCR in the laboratory of Dr Joseph Grippo (Hoffmann-LaRoche, Nutley, USA) and RAR-β, RAR-γ and RXR-γ were treated by PCR in the laboratory of Dr Peter LeMotte (Hoffmann LaRoche, Basle, Switzerland) using the original sequence information. A 2.2-kb *Eco*RI fragment of the mouse RXR-β (H-2RIIBP) cDNA [46] was provided by Dr K. Ozato (National Institutes of Health, Bethesda, USA). An 0.7-kb *Eco*RI fragment of a rat CRBP-I cDNA and a 0.7-kb *Eco*RI fragment of a rat CRABP-I cDNA were provided by Dr W.S. Blaner (Columbia University, New York). A 1.9-kb *Bgl*II fragment of the human t-PA cDNA [47], a 2.5-kb *Eco*RI fragment of a human PAI-1 cDNA of the 3.1-kb transcript [48], and a 1.2-kb *Pst*I fragment of a rat glyceraldehyde-3-phosphate dehydrogenase cDNA [49], which is commonly used as an internal standard probe [50] were provided by Dr R. Offringa (State University Leiden, The Netherlands). The cDNA fragments were radiolabelled using the random primer method (Multiprime, Amersham).

RESULTS

RAR and RXR mRNA expression

Northern-blotting studies were conducted to examine the expression of RAR and RXR mRNAs in cultured HUVEC before and after a 24-h exposure of the cells to 1 μM retinoic acid or the retinobenzoic acid, Ch55 [37,38] (Fig. 2). Two transcripts for RAR-α (≈3.6- kb and ≈2.8- kb) and two for RAR-γ (≈3.3-kb and 3.1-kb) were evident in the cells before and after the 24-h exposure to the retinoids (Fig. 2A). Detection of the RAR-γ transcripts required relatively long film exposure times, whereas an overnight exposure was usually sufficient to detect the RAR-α mRNAs. Very low levels of ≈3.4-kb and ≈3.2-kb RAR-β mRNA were detected in untreated cells, whereby the 3.2-kb transcript was very near the limits of detection of this Northern-blotting procedure. In contrast to the expression of the RAR-α and RAR-γ transcripts, the two RAR-β transcripts responded to retinoic acid and Ch55 with a 3-5-fold increase in expression compared to control levels. Induction of both the 3.4- kb and 3.2- kb RAR-β transcript was detected as early as 4 h after exposure of the cells to retinoic acid or Ch55; steady-state levels were reached after 12 h (results not shown).

The human RXR-α mRNA migrated as a single species of approximately 4.8-kb and displayed the strongest level of expression for the RXRs in HUVEC ; the expression of RXR-α was increased in the presence of retinoic acid and Ch55 (Fig. 2B). In contrast to the other RARs and RXRs, RXRα could only be specifically visualized in poly(A)-rich RNA, not in total RNA preparations. Only a very weak band for RXR-β (2.4-kb) was found, whereas no RXR-γ could be demonstrated, even after very long exposure times (6 weeks).

CRBP-I and CRABP-I mRNA expression

In the cytosol, retinoids may bind to the cellular retinol-binding or retinoic acid-binding proteins (CRBP and CRABP, respectively), which have been implicated in the storage, transport and/or metabolism of retinoids [51]. Furthermore, CR(A)BP may be responsible for the regulation of the free levels of retinoids for the ultimate action on the modulation of gene expression [52]. In HUVEC, one species of CRBP-I mRNA is expressed, with a size of approximately 0.7-kb (Fig 2C). Hybridization with the cDNA for CRABP-I revealed the presence of a 1.0-kb mRNA species. Treatment of HUVEC with 1 μM retinoic acid or Ch55 for 24 h induced CRBP-I but not CRABP-I gene expression.

t-PA and PAI-1 mRNA expression

For comparison, we also subjected the blots to hybridization with t-PA, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase cDNA probes. HUVEC incubated for 24 h in the presence of 1 μM retinoic acid or Ch55, contained about 3- 4-fold higher levels of t-PA mRNA than control cells, but showed little change in mRNA levels for PAI-1 or GAPDH (Fig 2D).

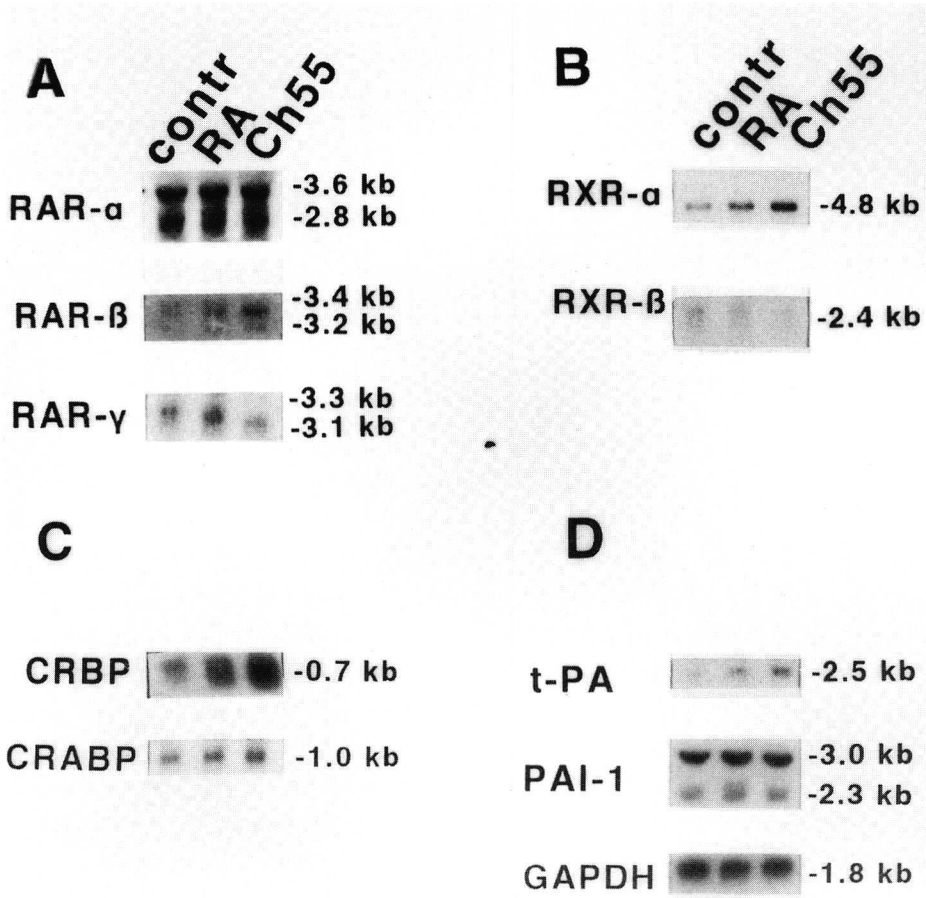

Fig. 2. mRNA expression of RARs, RXRs, CR(A)BP, t-PA and PAI-1 in HUVEC treated with retinoic acid or Ch55.

HUVEC were cultured in the absence or presence of retinoic acid or Ch55 (1 μM) for 24 h. Total cellular RNA (10 μg) or poly(A)-rich RNA (2.5 μg) in the case of RXR-α analysis were size-fractionated on a 1.2% agarose-formaldehyde gel and blotted onto a nylon filter. Four separate blots were first hybridized against the ^{32}P-labelled cDNA probes for RAR-β, RAR-γ, RXR-α, and t-PA mRNAs. After stripping, the blots were rehybridized with RAR-α, RXR-β, RXR-γ and CRBP-I cDNA probes, respectively. Finally, the blots were, after stripping, rehybridized with CRABP-I and PAI-1 cDNA probes. As a control for equal loading the blots were probed with the cDNA for glyceraldehyde-3-phosphate dehydrogenase mRNA. The autoradiograms for the RARs are shown in (A), those for the RXRs in (B), those for the CR(A)BP-Is in (C) and those for t-PA, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase in (D). The size of the various transcripts was calculated by comparison to the migration of a 0.24- to 9.5-kb RNA ladder (BRL).

RAR-subtype specific t-PA induction

Recently, a series of potent and novel synthetic retinoids named retinobenzoic acids [37,38] has been developed which exhibit different binding affinities for the different RARs [53,54]. We have used two of these synthetic retinoids, Ch55 and Am80, to elucidate the role of the multiple RAR subtypes in the induction of t-PA. In contrast to retinoic acid, Am80 and Ch55 have no or low binding affinity for RAR-γ and CRABP-I, respectively (Table 1).

Table 1. Comparison of binding activities of retinoic acid, Am80 and Ch55, as reported by Hashimoto [53].

Binding protein	Order of binding activity
RAR-α	Ch55 > retinoic acid > Am80
RAR-β	Ch55 > Am80 > retinoic acid
RAR-γ	Ch55 > retinoic acid (Am80, no binding)
CRABP	retinoic acid » Am80 (Ch55, no binding)

We have tested Ch55, Am80 and retinoic acid at concentrations of 10^{-12}-13 x 10^{-5} M for their effects on t-PA expression after 24, 48 and 72 h exposure. At higher test concentrations of the retinoids, HUVEC became detached from the matrix, which was accompanied by a fall in t-PA production. Incubations of longer than 24 h did not stimulate t-PA expression further (results not shown). The rank order of the retinoids, as determined by the retinoid concentration at which a half-maximal effect on t-PA expression is seen (ED_{50}), is Ch55 (0.5 nM) > retinoic acid (20 nM) > Am80 (80 nM) (Fig.3). The similarity between the potencies of the 3 retinoids to induce t-PA and the reported binding to RAR-α suggests that RAR-α plays a role in the t-PA induction process, assuming that Am80 has no affinity for RAR-γ [53]. If Am80 binds to RAR-γ with low affinity as reported by [55], the determined rank order of activity could also apply to a RAR-γ-mediated event. The latter possibility is unlikely, however, on the basis of the results obtained with Ro 13-7410 [p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl] benzoic acid][11]. In a transcription activation assay with RAR-α, the ED_{50} value for Ro 13-7410 has been reported to be lower than that for retinoic acid while, with RAR-γ, the ED_{50} for retinoic acid was lower [56]. We found out that the Ro 13-7410 concentration required to obtain half-maximal induction of t-PA in HUVEC is lower than that for retinoic acid (data not shown), which points to an involvement of RAR-α rather than RAR-γ in the t-PA induction by retinoids in HUVEC .

Our results also indicate that binding to CRABP is not a pre-requisite for the modulation of t-PA synthesis by retinoids, since Ch55 and Am80, which do not or only weakly compete with retinoic acid for binding to CRABP, are both effective inducers of t-PA synthesis. This does not necessarily imply, however, that CRABP-I plays no role at all in the regulation of t-PA by retinoids other than Am80 and Ch55 [52].

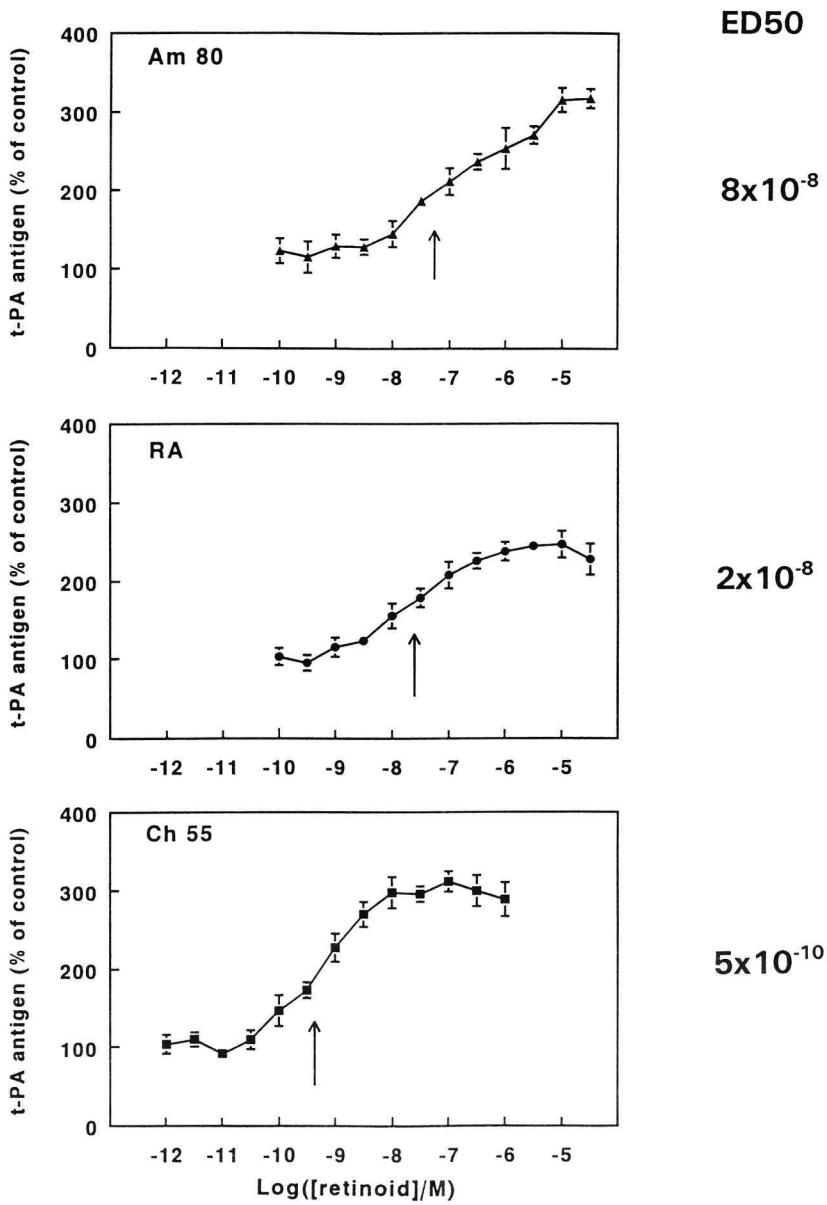

Fig. 3. Dose/response of retinoic acid, Ch55 and Am80 on t-PA antigen production in HUVEC.
HUVEC were incubated for 24 h with differing concentrations of Am80 (top), retinoic acid (middle) and Ch55 (bottom), and the conditioned media were analyzed for t-PA antigen. Results are means ± S.D. of three independent experiments, with incubations in duplicate; the data are expressed relative to the controls. The vehicle (Me_2SO) had no effect on t-PA at the concentrations used. The arrows indicate the ED_{50} for each of the retinoids tested.

Effect of Ro 41-5253, a RAR-α antagonist, on retinoid-stimulated t-PA synthesis

To further evaluate the putative role of RAR-α in mediating the induction of t-PA expression by retinoids, we have studied the effect of Ro 41-5253 on this process. Ro 41-5253 is a retinoid that antagonizes the transactivation of RARs by retinoic acid, having a preference for RAR-α [39,57]. Retinoic acid and Am580 have been used by Apfel et al. [39] to illustrate the antagonistic activity of Ro 41-5253 (see Table 2). The antagonist caused a dose-dependent reduction of t-PA induction, with the relative effective concentration of the antagonist depending on the potency and the concentration of the agonist. At the highest antagonist concentration (10 μM), the stimulation induced by 10 nM retinoic acid (Fig. 4A) or Am580 (Fig. 4B) could be largely suppressed. At 1 μM ligand concentration, 10 μM Ro 42-5253 did not antagonize the stimulation by retinoic acic anymore, but still could partly suppress the stimulation by Am80, reflecting the lower affinity of Am80 for RAR-α.

Table 2. Binding to RAR-α, RAR-β and RAR-γ of retinoic acid, Am580, and Ro 41-5253 (from [39]). IC_{50} retinoid concentration required to inhibit 50% of the specific retinoic acid binding.

Retinoid	(IC_{50}, nM) RAR-α	RAR-β	RAR-γ
Retinoic acid	14	14	14
Am580	39	870	2700
Ro 41-5253	60	2400	3300

These results are in line with the role of RAR-α. However, since the suppression by Ro 41-5253 was never complete, we cannot exclude the possibility that, especially at high retinoid concentrations (for example 1 μM retinoic acid), stimulation of t-PA expression also partially occurs via the other RAR subtypes. Remarkably, Ro 41-5253 also partly suppresses basal t-PA expression.Assuming that the antagonist action of Ro 41-5253 is restricted to RAR-α, this would imply that RAR-α is also involved in constitutive t-PA gene expression. Under all the above experimental conditions, PAI-1 synthesis remained within 10% of control values (results not shown).

RXR and 9-*cis* retinoic acid

The ligand for the RXRs has recently been shown to be 9-*cis* retinoic acid [29,58,59], which was found to be up to 40-fold more active than all-*trans* retinoic acid in controlling RXR activity, but about equipotent in activating RAR activity. Hence, under suboptimal stimulatory conditions, RXR activity can be induced specifically by 9-*cis* retinoic acid, whereas RAR activity is induced by both all-*trans* retinoic acid and 9-*cis* retinoic acid [29,58,59]. To assess the relative

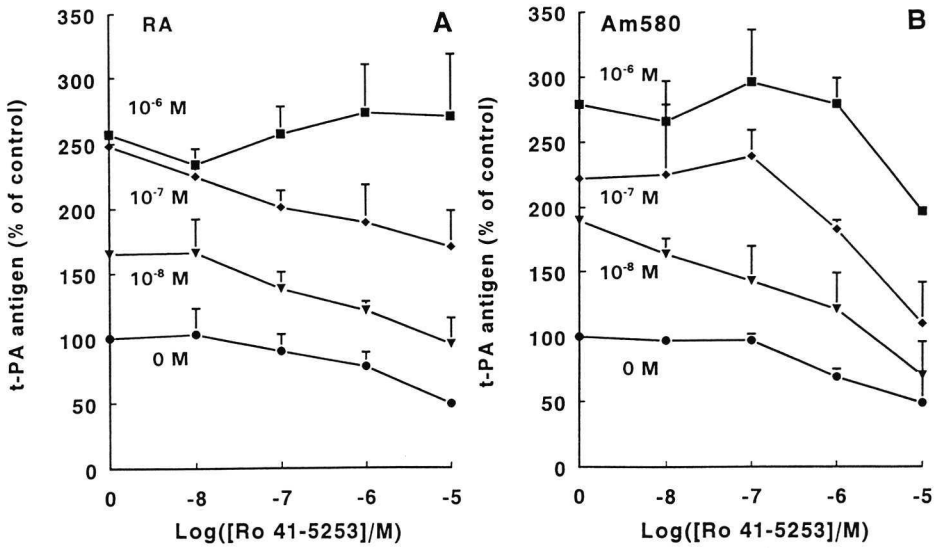

Fig. 4. Effect of Ro 41-5253, a RAR-α antagonist, on retinoic acid- and Am580-stimulated t-PA synthesis. HUVEC were incubated for 24 h with differing concentrations of retinoic acid (A) or Am580 (B). Simultaneously, the concentrations of the RAR-α antagonist, Ro 41-5253, indicated, were present in the incubation medium. Conditioned media were collected and analyzed for t-PA antigen. Results are means ± S.D. of three independent experiments, with incubations in duplicate; the data are expressed relative to the control values.

contribution of the RARs and the RXRs in the induction of t-PA expression by retinoids, we have compared the effect of 9-*cis* retinoic acid and all-*trans* retinoic acid alone or in combination on the synthesis of t-PA in HUVEC (Fig. 5). Both at suboptimal (10-100 nM) and saturating (1-10 μM) concentrations, 9-*cis* retinoic acid is less potent than all-*trans* retinoic acid in stimulating t-PA synthesis; this is more indicative of a RAR-mediated response rather than of a RXR-mediated response. Simultaneous addition of suboptimal concentrations of 9-*cis* and all-*trans* retinoic acid stimulated t-PA synthesis more than either compound alone, but never exceeded t-PA synthesis seen at maximally stimulating all-*trans* retinoic acid concentrations. This also suggests that ligand-bound RXR is not primarily involved in the signalling pathway for retinoid-stimulated t-PA synthesis. None of the 9-*cis* retinoic acid and all-*trans* retinoic acid concentrations, alone or together, showed a marked effect on PAI-1 production.

DISCUSSION

This report describes the expression of various RAR and RXR subtypes in cultured HUVEC, and provides evidence for a role of RAR-α in the retinoid-induced stimulation of t-PA gene expression in these cells. A role for RAR-α was concluded from a correlation between the t-PA stimulating activity of selective retinoids and their affinity for RAR-α. Also, a specific antagonist for RAR-α could interfere with the induction of t-PA by retinoids.

The mRNA patterns and the major transcript sizes of the RARs in HUVEC were found to be roughly similar to those previously reported for other cell types. HUVEC express two transcripts of RAR-α of approximately 3.6 kb and 2.8 kb (Fig. 2A). These two transcript sizes for RAR-α presumably reflect differences in polyadenylation and/or transcriptional termination [23]. Under basal, i.e. essentially retinoid-free, conditions, two RAR-β transcripts of approximately 3.4 kb and 3.2 kb were detectable at low concentrations in HUVEC. However, in the presence of 1 μM retinoic acid or Ch55, the levels of the two RAR-β mRNAs increased by a factor 3- 5 (Fig. 2A). Induction of RAR-β mRNA by retinoids has also been reported for several other types of cultured cells, including human hepatoma cells and mouse embryonal carcinoma cells [60-62]. *In vivo*, RAR-β mRNA is also greatly increased in lung (16-fold) and liver (7-fold) from retinol-deficient rats after refeeding with retinoic acid [63].

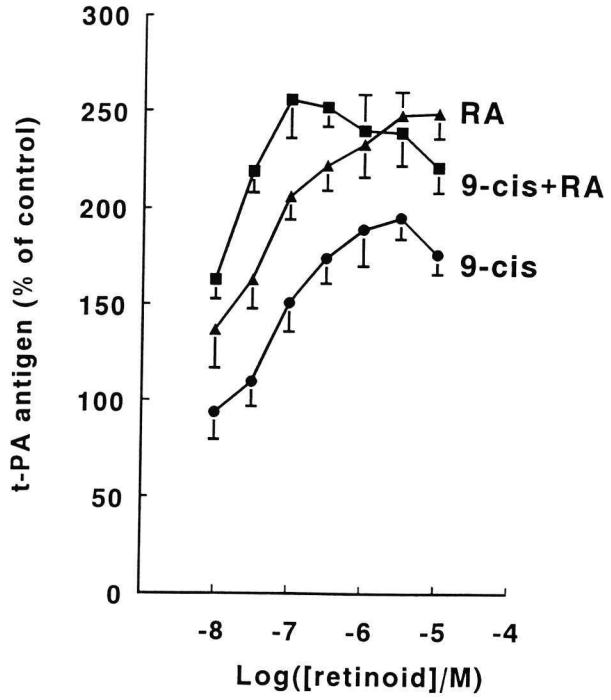

Fig. 5. Effect of all-*trans* retinoic acid and 9-*cis* retinoic acid alone and in combination on t-PA synthesis in HUVEC.
HUVEC were incubated for 24 h with differing concentrations of all-*trans* retinoic acid (RA) and 9-*cis* retinoic acid (9-cis), alone and in combination, and the conditioned media were analyzed for t-PA antigen. Results are means ± S.D. of four separate experiments, with incubations in duplicate; the data are expressed as relative to the control values.

Our results seem in conflict with the data reported by Fesus et al. [64], who showed that HUVEC contain high levels of mRNA for RAR-β. Also, no alterations in the expression of RAR-β transcripts were seen when retinoic acid was added to the cells. In contrast to our experiments, however, these workers cultured their endothelial cells in M199 medium. Since M199 medium contains relatively high concentrations (about 0.3 μM) of vitamin A acetate, their endothelial cells probably reflect retinoid-treated cells. The two closely spaced transcripts of 3.3-3.1 kb, detected with the RAR-γ probe in HUVEC, may well represent the two major RAR-γ isoforms [25], and became visible only after long exposure times (Fig. 2A).

Using retinoids with different affinity towards the various RAR subtypes or RXR, we found that the potency of these compounds to stimulate t-PA synthesis correlated with their affinity for RAR-α (Figs. 3 and Table 1). Further evidence for a role of RAR-α in the t-PA stimulatory activity of retinoids came from studies with a RAR-α selective antagonist, Ro 41-5253 (Fig. 4, Table 2). This retinoid, with selective binding to RAR-α, is not competent to activate the receptor in a transactivation assay. Similarly, as described by Apfel et al. [39,57] for the transactivation assay, an excess of antagonist over agonist was required for the inhibition of t-PA induction, which was inversely related to the potency of the various agonists to stimulate t-PA synthesis (Fig. 4). A role for RAR-α in t-PA induction by retinoids was also suggested by Espeseth et al. [65]. These investigators demonstrated that overexpression of an RAR-α, truncated in the ligand-binding domain in F9 cells, inhibited the retinoic acid-inducibility of t-PA without affecting the expression of Hox-1.3 and type IV collagen. The authors hypothesized that the transfected truncated receptors interfered with endogenous RARs in a negative dominant fashion. Although recent evidence suggests that RARs may bind more readily to their response elements as heterodimers with a member of the RXR family [28-31], our results obtained with 9-*cis* retinoic acid are not supportive of a quantitatively inportant role for a ligand-activated RXR in the induction of t-PA by retinoids (Fig. 5). Since HUVEC express RXR-α and RXR-β mRNAs (Fig.2B), we can not exclude the possibility, however, that RXR(s) are involved in transactivation in a ligand-independent way.

HUVEC express one species of CRBP-I mRNA (0.7 kb) and one species of CRABP-I mRNA (1.0 kb), which may play a role in channeling retinoid metabolism and in the delivery of sequestration of retinoids to or from the RARs/RXRs [51,52] . Our results indicate that t-PA-inducing retinoids need not bind to CRBP-I or CRABP-I in order to exert their effects; Ch55 [53,66] and 9-*cis* retinoic acid [67] show no affinity for the binding proteins, but are able to induce t-PA. This is in accord with the report that the induction of plasminogen activator production by several synthetic retinoids in F9 embryonal carcinoma cells can be correlated with their affinity for the nuclear retinoid receptors but not CRABP [68]. Also, some retinoid-responsive cell lines do not possess detectable levels of CRABP [69], indicating that CRABP is dispensable for gene regulation. This does not exclude, however, a role for CRBP and CRABP in the metabolism and regulation of the amount of intracellular retinoic acid and derivatives

thereof, and thus control quantitatively (rather than qualitatively) the intensity of the biologic effects [67].

Our findings on the role of RAR-α in t-PA gene expression are in line with the concept that each RAR isoform may control the expression of a specific subset of retinoic acid target genes (see also [70] for a review). Surprisingly, recent findings concerning RAR knock-out mice support the suggestion that there could be considerable functional redundancies amongst the various RARs [71,72]. However, to determine whether or not the apparent phenotypic redundancies truly reflect functional redundancies at a molecular level, it will be necessary to examine the expression of a variety of putative retinoic acid-responsive genes in the various RAR- knock-out mice [72].

To summarize, retinoids induce t-PA in HUVEC via a pathway that involves RAR-α. Further studies will be directed at elucidating whether or not RAR-α interacts directly with the t-PA promoter.

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CHAPTER 3

STIMULATION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR EXPRESSION BY RETINOIC ACID IN HUMAN ENDOTHELIAL CELLS REQUIRES RETINOIC ACID RECEPTOR ß2 INDUCTION

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ABSTRACT

We previously showed the involvement of retinoic acid receptor α (RARα) in the induction of tissue-type plasminogen activator (t-PA) synthesis by retinoic acid (RA) in human umbilical vein endothelial cells (HUVECs). However, the rather slow onset of this induction of t-PA synthesis suggested, however, an indirect role of RARα. Here we show that the protein synthesis inhibitor, cycloheximide (CHX) completely blocks the induction of t-PA by RA, which points to the need of an intermediary protein in t-PA stimulation. This intermediary protein is likely to be RARß2 on the basis of the following findings: (1) the induction of RARß by RA exactly precedes that of t-PA; (2) HUVECs with elevated RARß mRNA levels show an undelayed t-PA induction on stimulation with RA, and this response can be almost completely inhibited with an RAR antagonist; and (3) an antisense oligodeoxynucleotide against the translation initiation site of RARß2 mRNA greatly reduces the t-PA induction by RA. Thus, induction of t-PA by RA in HUVECs involves a 2-step mechanism requiring induction of RARß2 via RARα, followed by induction of t-PA synthesis via RARß2. Each of these steps is shown to have a different activation profile with RA and 9-*cis* RA.

INTRODUCTION

Several clinical studies have shown an inverse correlation between blood fibrinolytic activity and the risk of thromboembolic disease.[1,2] Consequently, much attention is being paid to the factors that govern blood fibrinolytic activity. Tissue-type plasminogen activator (t-PA), a key enzyme in the initiation of the fibrinolytic process, converts the zymogen plasminogen into the active enzyme plasmin.[3] Plasmin can degrade fibrin, the matrix structure of a blood clot, into soluble fibrin degradation products. The importance of the role of t-PA in plasma fibrinolysis has recently been emphasized once more by Carmeliet et al.[4], who showed that t-PA-deficient mice suffered an impaired thrombolytic potential in combination with an increased susceptibility towards endotoxin-induced thrombosis. The vascular endothelium plays an important role in determining plasma t-PA activity by synthesizing both t-PA and a specific inhibitor, plasminogen activator inhibitor-1 (PAI-1). We and others have previously shown that retinoids, i.e. vitamin A and derivatives, rather specifically stimulate t-PA synthesis in cultured human endothelial cells, without markedly influencing PAI-1 synthesis.[5-9] In vivo studies in rats also show that t-PA levels and retinoid status are correlated. [7,10] Because of their physiological relevance and their potential as a profibrinolytic drug we are interested in the mechanism(s) by which retinoids stimulate t-PA expression in human endothelial cells.

Many of the biological effects of retinoids are assumed to be mediated via nuclear

retinoid receptors. Two families of these receptors have been identified, the retinoic acid receptors (RARs) and the retinoid-X receptors (RXRs). Both RARs and RXRs are ligand-inducible transcription factors that modify the expression of specific genes by binding to specific DNA sequences, designated retinoic acid response elements (RAREs; for reviews, see Chambon[11]and Giguère[12]). Recently, Bulens et al. showed the presence of a RARE at position -7.3 kb in the t-PA promoter; cotransfection experiments in HT1080 cells showed that this RARE is functional.[13] Both RARs and RXRs consist of three subtypes, designated α,ß and γ, and because of alternative splicing and the use of different promoters, there are at least two different isoforms of each receptor subtype. Because RARs and RXRs function as either homodimers or heterodimers in binding to the RAREs, a great variety of homodimeric and heterodimeric combinations is possible. Each RAR/RXR subtype combination may specifically control the expression of a subset of RA target genes.[14]

The question arises whether the induction of t-PA by retinoids in human endothelial cells is also mediated via RARs/RXRs and, if so, which receptor subtype(s) is involved. In a previous report we showed by Northern analysis that cultured human umbilical vein endothelial cells (HUVECs) express all three RAR subtypes, RXRα and RXRß, albeit to a widely different extent.[9] Using subtype-specific ligands and an antagonist with a high preference for RARα, we could identify RARα to be involved in the induction of t-PA by retinoids in HUVECs. However, the rather slow onset of this induction left open the possibility that the role of RARα in t-PA expression is an indirect one. We now show that the induction of t-PA by RA in HUVECs is dependent on ongoing protein synthesis, suggesting that, besides RARα, a second (transcription) factor is required that is not present or is present at levels that are too low under basal conditions. By applying antisense technology, we showed that this factor is likely to be RARß2. Our data are consistent with a 2-step mechanism, in which RARα mediates the induction of RARß2, which subsequently mediates the induction of t-PA. Un further analysis, each of these steps turned out to have its own ligand-dependency characteristics for RA, which preferentially binds to RARs, and for 9-*cis* RA, which is a ligand both for RARs and RXRs.

MATERIALS AND METHODS

Materials

All-*trans* RA, cycloheximide (CHX), and dimethyl sulphoxide (DMSO) were purchased from Sigma (St Louis, MO). 9-*cis* RA and the RARα antagonist, Ro 41-5253, were kindly provided by Drs M. Klaus and C. Apfel (Hoffmann-LaRoche, Basel, Switzerland). Although Ro 41-5253 has a high preference for RARα, a large excess of Ro 41-5253 can also considerably reduce RARß activation.[15] Stock solutions of RA (10 mmol/L), 9-*cis* RA (10 mmol/L), Ro 41-5253 (10 mmol/L) and CHX (5 mg/mL) were prepared in DMSO and stored at -20 °C. Stock solutions were diluted with incubation medium to the final test concentrations immediately before the start of an experiment. All experiments involving retinoids were performed in subdued light, and the tubes containing the retinoid solutions were covered with aluminium foil. Sterile, pyrogen-free human serum albumin (20% wt/vol) was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service

(Amsterdam, The Netherlands). Enzyme immunoassay kits for determination of human t-PA antigen (Thrombonostika t-PA) and PAI-1 antigen ("Imulyse") were obtained from Organon Teknika (Boxtel, The Netherlands) and Biopool (Umeå, Sweden), respectively. Detroxycytidine 5[α-^{32}P] triphosphate was from Amersham (Buckinghamshire, UK). Other materials used have been specified in the methods described or in the relating references.

Cell culture experiments

Endothelial cells were isolated from human umbilical cord veins using the method of Jaffe et al.[16] The cells were cultured in fibronectin-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mmol/L HEPES (pH, 7.4), 10% (vol/vol) heat-inactivated newborn calf serum, 10% (vol/vol) human serum, 150 μg/mL endothelial cell growth supplement[17], 2 mmol/L L-glutamine, 5 IU/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. The medium was replaced every 2-3 days. Subcultures were obtained by trypsin/EDTsA treatment of confluent monolayers at a split ratio of 1:3. The cells were used for experiments at the first or second passage. Sixteen to 24 hours before the start of an experiment, the media were replaced with incubation medium (DMEM supplemented with 20 mmol/mL HEPES [pH, 7.4], 10% [vol/vol] human serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin]. Conditioned media were obtained by incubating cells at 37 °C with incubation medium containing the appropriate concentration of the test compound or stock solvent DMSO (final concentration, maximally 0.1% [vol/vol]). The conditioned media were centrifuged for 5 minutes at 8000 rpm in a Microfuge centrifuge to remove cells and cellular debris. Conditioned media were stored at -20 °C until use. The cells were washed with ice-cold phosphate buffered saline (0.15 mol/L NaCl/10 mmol/L Na_2HPO_4/1.5 mmol/L KH_2PO_4 [pH, 7.4]) and were used for isolation of RNA.

Antisense experiments

Ten hours before the start of an experiment, first-passage HUVECs were washed twice with DMEM and then incubated with DMEM supplemented with 20 mmol/L HEPES (pH, 7.4), 0.1% (wt/vol%) human serum albumin, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Subsequently, after a preincubation for 1 hour with 30 μmol/L antisense RARß2 (Fig 1B) or control (random or anti RARß4) oligo dTs (Fig 1B), the cells were stimulated with RA (final concentration 1 μmol/L) spiked into the medium; the amount of t-PA antigen produced during 12 hours was measured. To ensure the presence of a sufficient amount of antisense oligo dTs during the experiment, a second dose of oligo dTs (30 μmol/L) was added 4 hours after the addition of RA. Antisense oligo dTs were purchased from Isogen Bioscience (Amsterdam, The Netherlands) and were high-performance liquid chromatography-purified.

Northern blot analysis

Total RNA from HUVECs (75 cm^2) was isolated by the isothiocyanate/phenol/acid extraction method of Chomzynski et al.[18] The RNA was dissolved in H_2O and the RNA concentration was determined spectrophotometrically. Equal amounts (10 to 15 μg) of RNA were separated on a formaldehyde/agarose gel[19] and subsequently capillary transferred to a Hybond N membrane according to the instructions of the manufacturer (Amersham, Buckinghamshire, UK). Hybridization was performed in 7% (wt/vol) sodium dodecyl sulfate (SDS), 1 mmol/L EDTsA, 0.5 mol/L Na_2HPO_4/NaH_2PO_4 buffer, (pH, 7.2) overnight at 63°C with 25 ng of probe labeled with the random primer method (Megaprime kit, Amersham). The membranes were subsequently washed twice with 2xSSC/1%(wt/vol) SDS (1xSSC=0.15 mol/L NaCl, 0.015 mol/L Na_3citrate) for 20 minutes and once with 1x SSC/1% SDS for 20 minutes. The filters were exposed to an Amersham Hyperfilm-MP film with an intensifying screen at -80 °C.

cDNA probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.4-kb *Bam*HI-*Xba* I fragment of a human RARß cDNA, which was subjected to polymerase chain reaction (PCR) in the laboratory of Dr P. LeMotte, (Hoffmann-LaRoche, Basel, Switzerland); and a 1.9 kb *Bgl* II fragment of the human t-PA cDNA[20] and a 1.2 kb *Pst* I fragment of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr R. Offringa, State University Leiden, The Netherlands).

Assays

t-PA antigen and PAI-1 antigen determinations were performed by commercially available immunoassay kits. The enzyme immunoassay for determination of human PAI-1 antigen detects active and "latent" (inactive) forms of PAI-1, whereas t-PA/PAI-1 complexes are recovered with about 10-fold lower efficiency.

Reverse transcriptase-PCR (RT-PCR)

RT-PCR was performed under standard conditions following the specifications recommended by the supplier. The primers used are specified in Figure 1A. In short, RARß and actin cDNAs were synthesized in one reaction mixture containing 0.25 µg total RNA, 1.6 µg RARß cDNA primer (Fig 1A), 0.5 µg oligo dTs primer and RT-II-superscript (Life Technologies, Paisley, UK); then the cDNAs were heated for 8 minutes at 95 °C. Subsequently, the cDNAs were treated with RNAse H (25 U/mL) for 25 minutes at 37°C. Next, the RARß and actin cDNAs (1 µL of a 10x diluted cDNA reaction mixture) were amplified in the presence of 5% (vol/vol) DMSO and 5% W-1 (Life Technologies). The amplifications were performed for 30 cycles. The denaturation was performed during 20 seconds at 94 °C. Primer extension was performed for 90 seconds at 45 °C for the first 5 cycles and at 55°C thereafter. The DNA-synthesizing step was performed at 72°C for 3 minutes. Aliquots of the PCR reaction mixture were separated on an agarose gel, stained with ethidium bromide, and visualised with a UV transilluminator.

A

RARß cDNA primer 5'-TTTTTCTTGCATTTTAAATCCTGGAACTGAAGG-3'	3'-UTR RARß2/4 nt 1703-1735[55]
5' RARß2/4 PCR primer 5'-AACTTGGGATCTTTCTGGGAACC-3'	5'-UTR RARß2/4 nt 206-228[23]
3' RARß2/4 PCR primer 5'-CTGGGGAATGTTTGAAGTAGCTAG-3'	3'-UTR RARß2/4 nt 1677-1700[55]
5' human actin PCR primer 5'-AAGATGACCCAGATCATGTTTGAG-3'	5'-actin nt 428-451[56]
3' human actin PCR primer 5'-AGGAGGAGCAATGATCTTGATCTT-3'	3'-actin nt 1080-1103[56]

B

antisense RARß2 5'-CAGTCAAACATGATCTCCCTTG-3'	translation start site nt 311-332[55]
antisense RARß4 5'-GTGTTTCAATTGCATTTTCCAG-3'	translation start site nt 256-265/620-631[24]
control 5'-TAAATTTGTGTTCTTCGACTGC-3'	

C

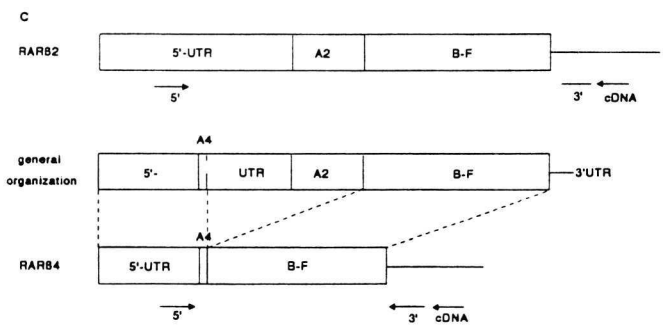


Fig 1. (A) Sequences of the primers used for RT-PCR of RARß and actin mRNA.
(B) Sequences of the antisense oligo dTs directed against the translation start site of RARß2 and RARß4 mRNA and sequence of the control oligonucleotide.
(C) Schematic representation (not to scale) of the general organisation of RARß cDNA and composition of the RARß2 and RARß4 cDNA isoforms (according to Nagpal et al.[24]). A2 represents the A domain of RARß2, and A4 represents the A domain of RARß4, which is a spliced variant of RARß2. The positions of the primers used for RT-PCR are indicated by arrows. "B-F" represent domains B-F of RARß. The RARß isoforms only differ in the 5'-untranslated region and the A domain; domains B-F and the 3'-untranslated region are common to all RARß isoforms.

Western blotting

For the detection of RARß protein, nuclear extracts were prepared from HUVECs as described previously by Andrews and Faller[21], with a few modifications. Briefly, cells were washed with phosphate-buffered saline and then allowed to swell on ice for 15 minutes in a buffer containing 10 mmol/L HEPES (pH, 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, and a mixture of proteinase inhibitors (leupeptin, aprotinin, pepstatin, antitrypsin and chymostatin) at a final concentration of 5 µg/mL. Next the cells were lysed by pushing them through a needle and then were centrifuged at 13,000 rpm in a Microfuge centrifuge for 30 minutes. After resuspending the nuclei pellet in a buffer containing 20 mmol/L HEPES (pH, 7.9), 25% (vol/vol) glycerol, 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTsA, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 0.5 mmol/L dithiothreitol, and the mixture of proteinase inhibitors (final concentration, 5 µg/mL), the suspension was rotated for 30 minutes at 4°C. Finally, the membranes were pelleted by centrifugation for 5 minutes at 13,000 rpm in the Microfuge centrifuge, and the supernatant was collected. The protein concentration of the supernatant was determined with the Bradford protein assay (Biorad, München, Germany). Aliquots containing 6 µg of protein were subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis under reducing conditions using a 7.5 % (wt/vol) polyacrylamide gel. Proteins were transferred to nitrocellulose electrophoretically using a semi-dry blotting device. Next the blots were stained with "Ponceau" (Sigma) to check for equal loading and blotting efficiency. The blots were incubated with rabbit polyclonal antibodies raised against the human F region of RARß [RPß(F)2; antibodies kindly provided by Prof P. Chambon, Strasbourg, France). As a second antibody, goat antirabbit antibody (Nordic, Tilburg, The Netherlands) conjugated to horse radish peroxidase was used. Detection was performed using the enhanced chemiluminescent detection method (Amersham) according to the manufacturer's instructions.

RESULTS

Onset of t-PA and RARß mRNA induction by RA

Figure 2 shows one representative time course of the three performed t-PA and RARß mRNA induction in HUVECs treated with 1 µmol/L RA for various periods up to 12 hours. t-PA mRNA levels start to increase after about 8 hours, reaching a 3-fold increase over control levels after 12 hours. Before any exposure to RA, HUVECs express very low levels of two transcripts, 3.2 and 3.4 kb, for RARß. Both transcripts are already increased 6.5- and 7.5-fold, respectively, already after 4 hours of incubation with RA and continue to increase to about 11- and 8-fold stimulation, respectively after 12 hours. As in many other cell types, the expression of RARα, RARγ, RXRα and RXRß in HUVECs did not change appreciably on incubation with RA (see also Kooistra et al[9]).

Effect of protein synthesis inhibition on the induction of t-PA and RARß mRNA by RA

To assess the importance of ongoing protein synthesis on the induction of t-PA mRNA by RA in HUVECs, cells were stimulated with 1 µmol/L RA in the presence of the protein synthesis inhibitor, CHX (5 µg/mL; added 1 hour before the start of the experiment). After 8 hours total RNA was isolated and analyzed by Northern blotting. As shown in Fig. 3, CHX (which inhibited protein synthesis up to about 95%; data not shown) completely blocked the induction of t-PA mRNA by RA. In contrast, CHX did not prevent the RA-stimulated increase in RARß mRNA. The fact that the induction of RARß by RA is rapid and CHX-resistant is in agreement with findings in other cell types in which it was shown that induction of RARß

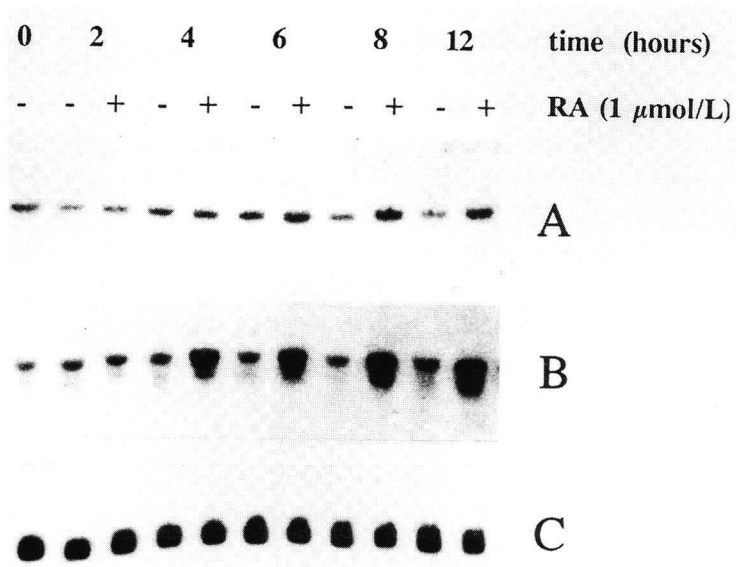


Fig 2. Time course of t-PA and RARß mRNA induction by RA. HUVECs were incubated for the indicated time periods with 1 μmol/L RA (+) or RA vehicle, 0.01% (vol/vol) DMSO(-). RNA was isolated and analyzed by Northern blot analysis (15μg/lane) using ^{32}P-labeled probes for t-PA (A), RARß (B) and GAPDH (C).

by RA is directly mediated by RARα.[22] The induction of t-PA apparently requires the synthesis of an intermediary regulatory protein, which explains the slower onset of the t-PA mRNA increase as compared with that of RARß mRNA.

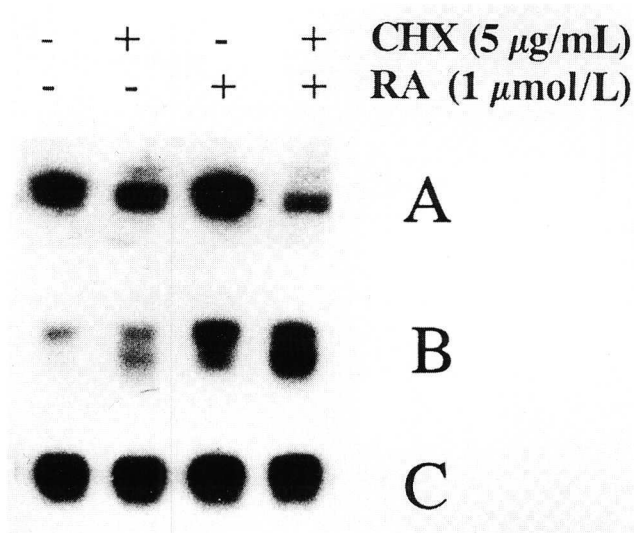


Fig 3. Effect of protein synthesis inhibition on the induction of t-PA and RARß mRNA by RA. Cells were incubated with or without RA (1μmol/L) in the absence or presence of CHX (5μg/mL). CHX was added 1 hour before; control medium contained 0.01% (vol/vol) DMSO. After 8 hours RNA was isolated and analyzed by Northern blot analysis using ^{32}-P labelled probes for t-PA (A), RARß (B) and GAPDH (C). This is a representative experiment out of four performed.

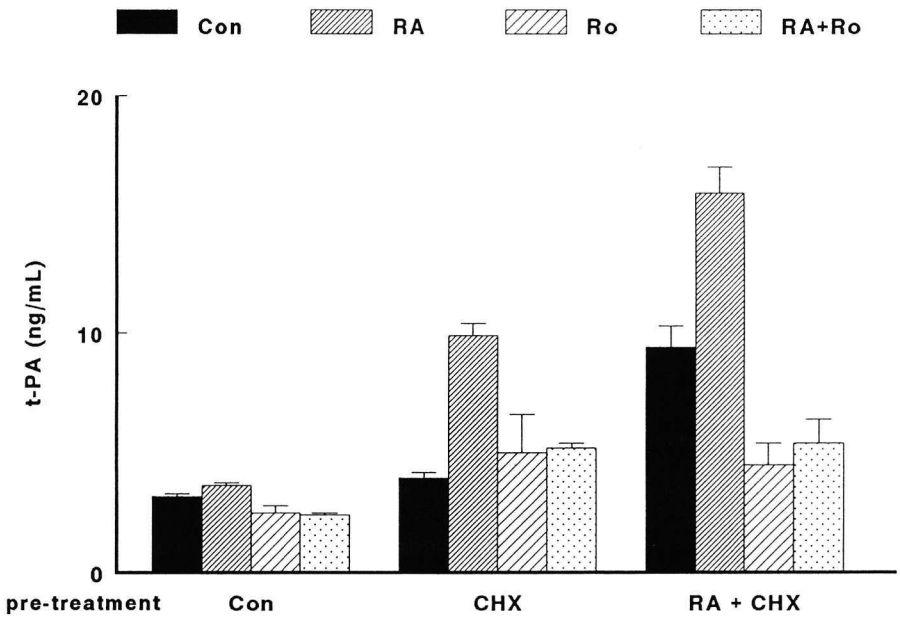


Fig 4. Effect of RA on t-PA antigen production in cells with elevated RARß mRNA levels and in the presence or absence of RAR antagonist (Ro 41-5253). HUVECs were pretreated for 8 hours with control medium (Con) (0.1% [vol/vol] DMSO), or with CHX (5µg/mL) and RA (1µmol/L), or CHX alone (5µg/mL) to increase RARß mRNA levels. After washing the cells 3 times with DMEM to remove residual CHX and/or RA, HUVECs were incubated for 8 hours with control medium (0.1% [vol/vol] DMSO;Con), 10nmol/L RA (RA), 10µmol/L Ro 41-5253 (Ro) or 10nmol/L RA and 10µmol/L Ro 41-5253 (RA + Ro). t-PA antigen levels in the conditioned media were determined with the t-PA enzyme-linked immunosorbent assay as described in Materials and Methods. The data shown are from one representative experiment of three performed and are expressed as mean values of a duplicate experiment, with ranges indicated by error bars.

Role of RARß in t-PA induction

Because the induction profile of RARß preceded that of t-PA (Fig 2), we examined the possibility that the intermediary factor postulated above is RARß. In a first approach to verify this hypothesis we designed the following experiment. HUVECs were pretreated for 8 hours with 5 µg/mL CHX and 1 µmol/L RA. This will lead to an increase in RARß mRNA levels without affecting t-PA mRNA levels (see Fig 3). If RARß is truly involved in t-PA gene expression, subsequent incubation of these pretreated cells with RA in the absence of CHX should induce t-PA synthesis much faster than it would do in control cells, in which RARß first needs to be induced. Moreover, this undelayed t-PA induction in the pretreated cells should be suppressible with an RAR antagonist. As shown in Fig 4 for one representative experiment of three performed, the addition of 10 nmol/L RA to the RA/CHX pretreated cells resulted in a 5-fold increase in t-PA synthesis during an 8 hour incubation period, whereas this RA concentration had a negligible effect on t-PA production in HUVECs preincubated

with control incubation medium. This undelayed t-PA induction by RA in the RA/CHX pretreated HUVECs could be largely suppressed by the simultaneous addition of 10 μmol/L Ro 41-5253, a retinoid that antagonizes the transactivation of RARs by RA.[15] Ro 41-5253 also strongly quenched the t-PA production in RA/CHX pretreated HUVECs that were subsequently incubated with control medium, suggesting that the relatively high t-PA production by these cells might be caused by residual RA from the pretreatment period. Therefore, we also evaluated t-PA synthesis in HUVECs pretreated with 5 μg/mL CHX alone, a condition also shown to increase RARß mRNAs, albeit to a lesser extent than that in combination with RA (Fig 3). In this case, incubation of the pretreated cells with control medium hardly affected t-PA synthesis; on addition of 10 nmol/L RA, a qualitatively similar response was observed as that described above for the RA/CHX pretreated cells, ie, a 2.5-fold increase in t-PA production that could be suppressed to a large extent by 10 μmol/L Ro 41-5253. Taken together, these results are consistent with a role of RARß as a mediator of t-PA induction by RA in HUVECs.

Identification of RARß isoforms

To find more conclusive evidence for a role of RARß in the stimulation of t-PA synthesis by RA, we wished to lower specifically RARß mRNA levels and translation by using antisense oligo dTs. Therefore, it was necessary to identify the RARß isoforms expressed in HUVECs. As shown in Fig 3, RA treatment of HUVECs induces 2 mRNA transcripts of sizes 3.4 and 3.2 kb in size, respectively. Western blot analysis showed one specific RARß protein band with a molecular weight comparable with that of RARß expressed in transfected COS cells (about 55 kDa); the intensity of the RARß band increased after RA treatment of HUVECs (Fig 5A). Four different isoforms of RARß are known, which only differ in their N-terminal A domain.[23,24] In contrast to RARß1 and RARß3, RARß2 and RARß4 (a spliced variant of RARß2) contain an RA-responsive promoter[22,24,25], suggesting that the two induced RARß transcripts in HUVECs are the ß2 and/or ß4 isoforms. Application of RT-PCR analysis, which is able to distinguish between the different isoforms (see Fig 1C), showed predominantly the presence and induction of RARß2 mRNA in HUVECs and did not show, or showed only to a minor extent, the presence of RARß4 mRNA (Fig 5B). Therefore, the two inducible RARß bands observed by Northern blot analysis are thus likely to predominantly represent two RARß2 transcripts.

Inhibition of RA-stimulated expression of t-PA by an antisense oligodeoxynucleotide against RARß2 mRNA

Because RARß2 mRNA appeared to be the major form induced by RA in HUVECs, we chose to inhibit RARß levels by an antisense oligodeoxynucleotide directed specifically against the translation start site of RARß2 mRNA, following the protocol described in the

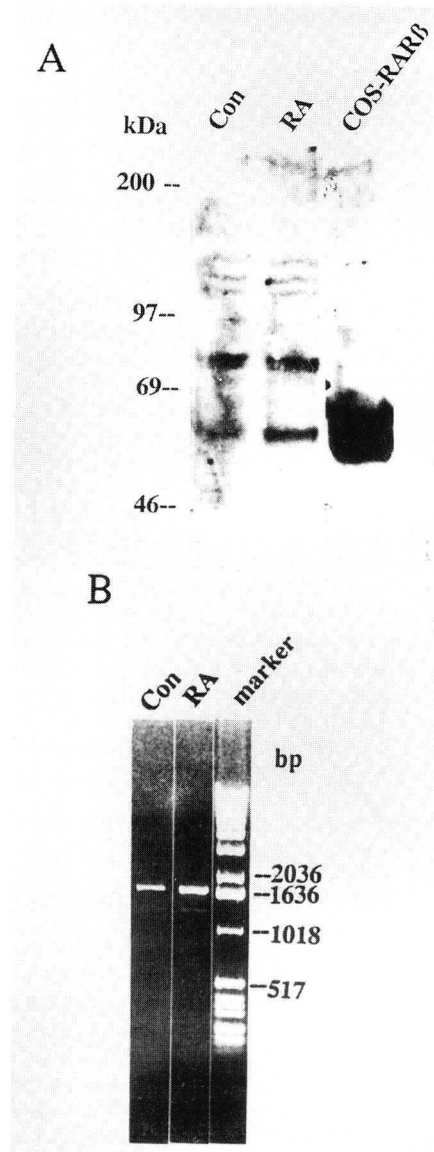


Fig 5. (A) Western blot analysis of RARß protein in control and RA-treated cells. HUVECs were incubated for 12 hours with vehicle (0.01% [vol/vol] DMSO) or RA (1µmol/L). Nuclear extracts were prepared from the cells as described in Materials and Methods. As a positive control nuclear extracts of COS cells transfected with a RARß expression vector were used. Equal amounts (6 µg) of endothelial nuclear extracts were subjected to electrophoresis on a 7.5% polyacrylamide gel, transferred to nitrocellulose, incubated with a polyclonal antibody against RARß, and followed by incubation with a second (goat anti-rabbit) antibody conjugated to horseradish peroxidase. Bands could be visualized by adding substrate. The RARß protein band is visible at 55 kD. The (slower migrating) intense band and some of the minor bands were also observed with higher concentrations of COS cells with or without a RARß expression plasmid and are considered to be aspecific. One representative experiment out of three performed is shown.
(B) Induction of RARß2 and RARß4 mRNA by RA determined by RT-PCR. After incubation of HUVECs for 12 hours with vehicle (0.01% [vol/vol] DMSO) or RA (1µmol/L), RNA was isolated. RARß cDNA was synthesized using 0.25µg total RNA and a specific RARß primer (see Fig 1A) as described in Materials and Methods. The cDNAs were amplified for 30 cycles using the sense primer
5'-AACTTGGGATCTTTCTGGGAACC-3'
and antisense primer
5'-CTGGGGAATGTTTGAAGTAGCTAG-3'
(see also Fig 1A) (20 seconds at 94°C, 90 seconds at 45°C in the first 5 cycles and at 55°C in the last 25 cycles, 3 minutes at 72°C). The expected length of the PCR fragment of RARß2 is 1641 nucleotides and that of RARß4 is 1286 nucleotides. The data presented are representative of four independent experiments.

Materials and Methods section. Besides blocking translation, binding of such oligo dTs to RARß2 mRNA molecules will make these molecules susceptible to degradation by RNAse H, which will result in a reduction of RARß2 mRNA levels. Figure 6 shows a typical experiment in which antisense oligo dTs directed against RARß2 at a concentration of 30

μmol/L effectively reduced RARß2 mRNA levels, as measured by RT-PCR, whereas a control random oligodeoxynucleotide (Fig 1B) did not affect RARß2 mRNA levels. The expression of actin mRNA was included to check for equal efficiency of the RT-PCR procedure between the different samples. As shown in Fig 7A, antisense RARß2 oligo dTs greatly (about 75%) inhibited the increase of t-PA synthesis by RA in HUVECs, whereas random and anti-RARß4 oligo dTs did not affect t-PA induction. As a further control on the specificity of the antisense RARß2 treatment, PAI-1 production was determined. PAI-1 synthesis appeared not to be affected by any of the antisense oligo dTs used (Fig 7B). Between experiments with different HUVECs isolates, the efficacy of the antisense oligo dTs directed against RARß2 to reduce RARß2 mRNA levels varied. The reason for this variation is not known at present but may be related to differences in uptake and/or stability of the antisense oligo dTs in different endothelial cell cultures. However, the decrease in t-PA synthesis correlated to the extent of RARß2 suppression (data not shown). Together, these results indicate that the induction of t-PA by RA in HUVECs can be specifically suppressed by using antisense oligo dTs against RARß2.

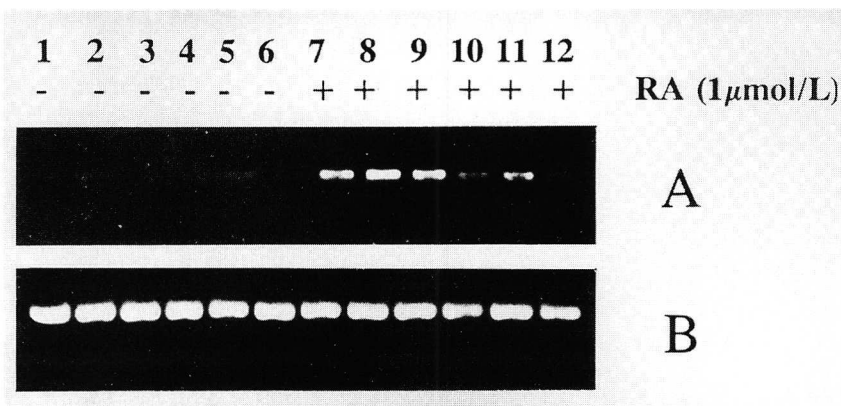


Fig 6. Effect of antisense oligo dTs directed against RARß2 on RARß2 mRNA levels as determined by RT-PCR. RNA was isolated from the cells, and cDNAs were made as described in Materials and Methods. RARß2 (A) and actin (B) cDNAs were amplified by PCR using the primers as shown in Fig 1A. Lanes 1-6 represent untreated cells, and lanes 7-12 are RA-stimulated (1 μmol/L) cells. The following additions were made, with the final concentration in brackets. Lanes 1 and 7: none, lanes 2 and 8, random oligo dTs (30μmol/L); lanes 3 and 9, antisense RARß4 oligo dTs (30 μmol/L); lanes 4 and 10, antisense RARß2 oligo dTs (30μmol/L); lanes 5 and 11, random oligo dTs (60 μmol/L); lanes 6 and 12, antisense RARß2 and RARß4 oligo dTs (both 30μmol/L).

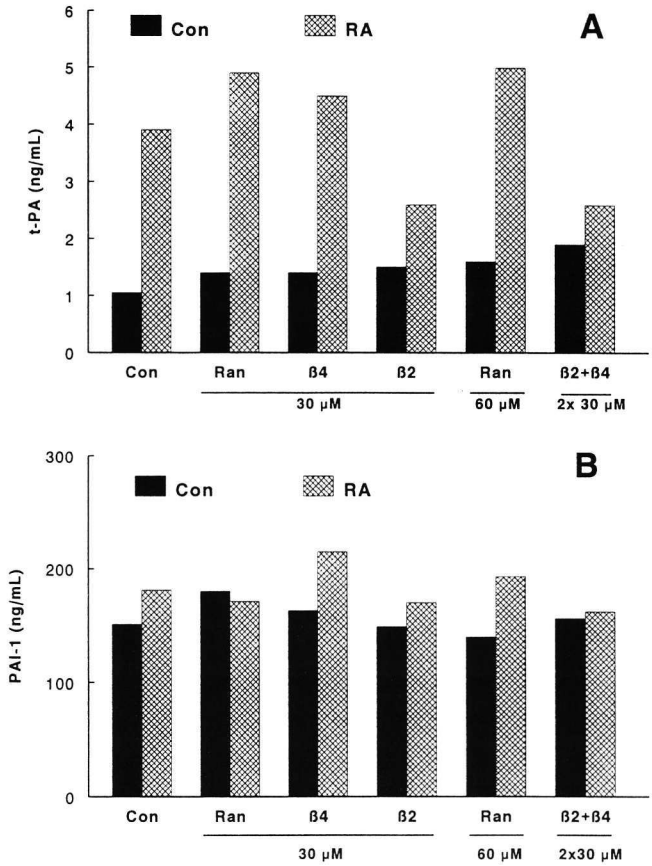
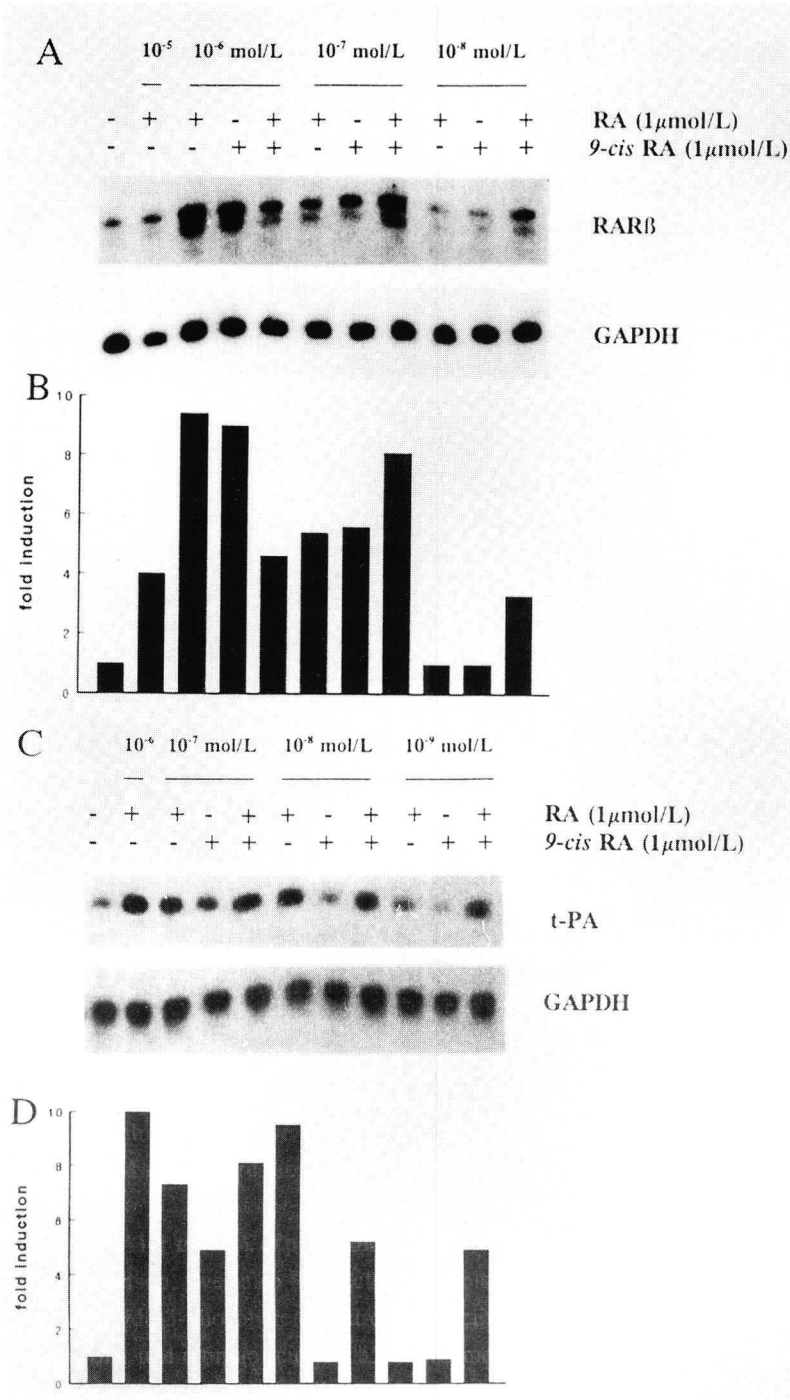


Fig 7. Effect of antisense oligo dTs directed against RARß2 mRNA on the induction of t-PA and PAI-1 synthesis by RA. HUVECs were incubated with or without RA (1 µmol/L) in the presence of antisense oligo dTs directed against RARß2 (ß2) and/or RARß4 (ß4) at the indicated concentrations. As controls medium with (Ran) or without (Con) random antisense oligo dTs was used. Oligo dTs were added 1 hour before and 4 hours after the addition of RA at the indicated concentrations. After 12 hours, t-PA (A) and PAI-1 (B) levels in the conditioned media were measured with the enzyme-linked immunosorbent assay procedures as described in Materials and Methods.

Fig 8. (A) Induction of RARß by RA, 9-*cis*-RA, or a combination thereof. HUVECs were incubated for 8 hours with the indicated concentrations of retinoids. RNA was isolated and analyzed (15 µg/lane) by Northern blot analysis using ^{32}[deoxycytidine triphosphate]-labeled probes for RARß and GAPDH. **(B) Quantification of RARß mRNA.** The signal intensity of the Northern blot was determined by scanning and normalized to the signal from the internal standard GAPDH. The amount of RARß mRNA is expressed relative to the amount of RARß mRNA in cells incubated with control medium.
(C) Stimulation of t-PA mRNA synthesis by RA, 9-*cis* RA, or a combination thereof in cells with elevated RARß levels. HUVECs were preincubated for 8 hours with CHX (5 µg/mL) to increase RARß mRNA levels. After washing the cells 3 times with DMEM, the cells were stimulated for another 8 hours with the indicated concentrations of RA, 9-*cis* RA, or both compounds. Total RNA was isolated and hybridized (15 µg/lane) to the ^{32}[deoxycytidine triphosphate]-labeled t-PA and GAPDH probes. **(D) Quantification of t-PA mRNA. The signal intensity of the Northern blot was determined by scanning and normalized to the signal from the internal standard GAPDH.** The amount of t-PA mRNA is expressed relative to the amount of t-PA mRNA of CHX pretreated cells incubated with control medium.


A
10⁻⁵ 10⁻⁶ mol/L 10⁻⁷ mol/L 10⁻⁸ mol/L
RA (1μmol/L)
9-cis RA (1μmol/L)
RARß
GAPDH
B
fold induction
C
10⁻⁶ 10⁻⁷ mol/L 10⁻⁸ mol/L 10⁻⁹ mol/L
RA (1μmol/L)
9-cis RA (1μmol/L)
t-PA
GAPDH
D
fold induction

Ligand dependency of the RARα and RARß mediated steps in t-PA induction

We next addressed the question of whether each of the two steps putatively involved in t-PA induction by RA, namely induction of RARß via RARα followed by t-PA induction via RARß, requires different ligand concentrations for activation. To that end, we assessed the relative potencies of RA, 9-*cis* RA and combinations thereof on the induction of RARß2 mRNA in control HUVECs and on the induction of t-PA mRNA in cells with elevated RARß levels by pretreatment with CHX. As shown in Fig 8A, for an 8 hour incubation period, RA and 9-*cis* RA were equipotent in inducing RARß mRNA; both compounds required a minimal concentration of 100 nmol/L to induce RARß mRNA, and induction levels increased to about 9-fold stimulation at 1 µmol/L with both ligands. A combination of the two isomers showed at least an additive effect on the expression of RARß mRNA at suboptimal concentrations, but never exceeded the maximal stimulation induced by either RA or 9-*cis* RA alone. At supramaximal concentrations (10 µmol/L RA or a combination of 1 µmol/L RA and 1µmol/L 9-*cis* RA) RARß mRNA induction levels decreased again. In contrast to the induction of RARß, RA had already induced t-PA mRNA already strongly at concentrations as low as 10 nmol/L in CHX pretreated cells, and this response was only slightly more increased at concentrations of 1 µmol/L (Fig 8C). 9-*cis* RA was much less effective than RA. At least 10-fold higher concentrations of 9-*cis* RA were required to achieve comparable induction of t-PA mRNA. In general, RA in combination with 9-*cis* RA was not more effective than RA alone in enhancing t-PA mRNA induction. These results indicate that each of the two steps involved in t-PA mRNA induction responds differently to (combinations of) different concentrations of RA and 9-*cis* RA, and that RA and 9-*cis* RA are not equipotent in inducing t-PA expression.

DISCUSSION

In a previous study, we have reported on the involvement of RARα in the induction of t-PA gene expression by RA in HUVECs.[9] However, the relatively slow action of RA on t-PA gene expression suggested a mechanism in which the induction of t-PA is a secondary response to activation of RARα by RA. We now provide evidence that a second step is indeed necessary and involves the synthesis of RARß2 on the basis of the following findings: (1) the kinetics of the RA-associated increase in RARß mRNA are consistent with the subsequent involvement of RARß in the regulation of t-PA; (2) the effect of RA on the induction of t-PA, but not that of RARß, was completely abolished by the protein synthesis inhibitor, CHX; (3) HUVECs with elevated RARß mRNA levels as a result of pretreatment of the cells with RA and/or CHX showed an undelayed increase in t-PA expression by RA; that could be quenched by the RAR antagonist Ro 41-5253; and (4) antisense oligo dTs directed against RARß2 greatly suppressed the induction of t-PA by RA. Taken together,

these results point to a 2-step mechanism in which first RARß2 is induced via RARα and then RARß2 mediates the induction of t-PA. Experiments with different concentrations of RA and 9-*cis* RA showed that each step has a different ligand dependency.

Complementary to our studies, Bulens et al.[13] identified a functional RARE in the t-PA promoter, consisting of a direct repeat of the GGGTCA motif spaced by 5 nucleotides (DR5) and localized at -7.3 kb.[13] Transient expression of a 2 kb RARE-containing t-PA promoter:CAT construct into the hybrid endothelial cell line EA.hy926 resulted in a 6-fold induction by RA, whereas no induction was observed with a reporter construct in which the t-PA DR5 RARE was eliminated by site-specific mutagenesis. A maximal induction by RA of this RARE containing t-PA promoter:CAT construct in the endothelial cell line required cotransfection with RARß and RXRα expressing plasmids. Our results in combination with those of the study by Bulens et al.[13] provide strong evidence that RARß2 is the ultimate mediator of t-PA induction by RA in HUVECs.

A 2-step mechanism as described above for the induction of t-PA by RA in HUVECs is not unique in itself for retinoid-induced genes. For example, similar observations were made with some retinoid-responsive genes in F9 cells, such as laminin B1, collagen IV, and J6 genes.[26] These genes were induced after a relatively long (12 to 24 hours) exposure to retinoids and their induction was also sensitive to CHX. Also, the relatively slow action of RA on transferrin and albumin gene expression in Hep3B cells (8 to 24 hours) may be a secondary response to RA.[27] Several factors have been shown to serve as an intermediary protein in the RA response, including c-jun, GATA-binding proteins, and NFκB heterodimers.[28-30] However, the number of RA-responsive genes whose induction is known to be mediated by a RAR as a secondary protein, such as the t-PA induction in HUVECs in this study, is still rather limited. To our knowledge, only the induction of major histocompatability complex class 1 in NTera-2 embryonal carcinoma cells has been shown to be at least partly dependent on the RA-induced increase of RARß.[30]

Our results do not exclude the possibility that, in addition to RARß2, other factors are required for the induction of t-PA by RA. Several reports have provided evidence that members of the steroid/thyroid receptor superfamily, including retinoid receptors, require cofactors for transcription activation. For example, Folkers and Van der Saag have shown that the adenovirus E1A protein functions as a cofactor for RARß mediated activation of transcription.[31] Also, functional interactions of peroxisome proliferator-activated receptor, RXR, and SP1 in the transcriptional regulation of the acyl coenzyme A oxidase promoter have been shown.[32] Whether RARß2 also requires a cofactor for induction of t-PA by RA is uncertain. However, it may be significant, as discussed by Bulens et al.[13] that, in the vicinity of the RARE in the t-PA promoter, several SP1 and AP2 consensus binding sites are present.

Our results support initial suggestions that the various RAR subtypes may preferentially control the transcription of different subsets of RA-responsive genes. This

concept was based on two types of observations: (1) the striking interspecies amino acid sequence conservation of a given RAR (and RXR) isoform, which is much higher than the similarity between the three RAR (or the three RXR) isoforms within a given species[11]; and (2) the specific spatio-temporal patterns of expression for each RAR (and RXR) gene as shown by in situ hybridization studies on embryo sections.[33-35] More direct evidence came from cotransfection experiments in which each of the RAR and RXR isoforms showed some specificity towards several synthetic and natural RARs.[36] Also, in a different type of study, targeted disruption of RARα in F9 cells resulted in reduced CRABPII and Hoxb1 mRNA expression in RA-stimulated cells.[37] However, it has been found that a certain degree of redundancy may exist in the retinoid-signaling pathway. For example, knock out mice for all RARα isoforms showed a phenotype that is relatively discrete considering the ubiquitous expression of RARα[38], and knock-out mice for specific isoforms studied so far (RARα1, RARß2 and RARγ2) have no apparent phenotype.[39-41] Similarly, some of the natural promoters that have been tested in co-transfection experiments could be activated, albeit to different extents, by more than one RAR or RXR form.[42] Also in the case of t-PA, Bulens et al.[13] showed that RARα, RARß, and RXRα were all able to stimulate a 2.4 kb RARE-containing t-PA promoter fragment linked to a CAT reporter gene in cotransfection experiments in HT1080 fibrosarcoma cells, although RARß in combination with RXRα gave maximal stimulation in these experiments.[13] However, in these studies transfected cells have been used expressing concentrations of RARs/RXRs at nonphysiologically high levels. Therefore, these transfection experiments therefore do not necessarily imply that the same regulatory mechanisms hold true under physiological conditions; ie, restricted receptor expression is one mechanism of specifying gene activation by hormones. Our results obtained in HUVECs indicate that, in a physiological context, RARα specifically induces RARß2 and that the RAR subtype ß2 is involved in the induction of t-PA, which points to a preference of different RAR subtypes to induce different target genes.

It is assumed that, in the cellular context, RARs act predominantly as heterodimers with RXRs.[43] Our results shown in Fig 8 show that RA and 9-*cis* RA are equipotent in stimulating RARß expression in HUVECs and that a combination of both ligands never is more effective that each compound alone at optimal concentration. For the induction of t-PA in HUVECs with elevated RARß levels, RA is even much more effective than 9-*cis* RA. Because RA acts mainly via binding to RARs, whereas 9-*cis* RA can bind to and activate both RARs and RXRs[44-46] ligand binding to RXR, if occurring, does not substantially contribute to the two transactivation steps. In line with this conclusion, we found that 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphtyl)ethynyl]benzoic acid, a compound shown to be RXR-specific[47], was only a very weak inducer of RARß and t-PA mRNA in HUVECs, (Lansink and Kooistra, unpublished data). A similar conclusion was reached by Xiao et al,[48] who showed that binding of a RXR specific ligand, SR11237, to RXR in RAR-

RXR heterodimers did not confer ligand-dependent transactivation of a ß-RARE-tk-CAT promoter construct.

A remarkable finding was the observation that higher concentrations of RA are required for the stimulation of RARß expression, which involves RARα activation, than for the induction of t-PA expression, which involves RARß activation. Binding studies with purified RARα and RARß preparations show equal affinity of both receptors for RA.[46,49] In agreement with our findings, Brand et al.[50] also reported a 10 times higher apparent affinity of RA for RARß than for RARα in a cellular system. A similar discrepancy between data from binding studies with purified receptors and data from cell studies exists for RA and 9-*cis* RA in relation to RARß. Our results show that 9-*cis* RA is less potent in inducing t-PA than is RA, although binding studies using isolated receptors show similar binding affinities of RA and 9-*cis* RA for RARß.[46,49] Apparently, cellular factors influence the binding affinities of retinoids for their receptors.

Our observation that RARß transcripts in HUVECs are upregulated by retinoid treatment is not just a cell culture phenomenon, because both a 3.3 kb and a 3.0 kb RARß mRNA are increased within 1 to 4 hours in lung and liver tissues from retinol-deficient rats receiving a single dose of RA (100 μg).[51] However, under normal physiological conditions, RA concentrations in plasma and tissues are sufficient to maintain a steady RARß expression.[52] Also, because of the high affinity of RARß for RA (Brand et al[50] and this study), it seems that the capacity of RA to stimulate t-PA synthesis in vivo is almost fully exploited under normal physiological conditions. In line with this finding, plasma t-PA activity in vitamin A-deficient rats is about 3-fold lower than control values, whereas RA treatment of normal rats increases plasma t-PA activity maximally by only 50%.[10] Similarly, treatment of male volunteers with isotretinoin (13-cis RA), which has been shown to induce t-PA levels in HUVECs[7], did not effect or only minimally effect t-PA antigen in plasma.[53,54] Therefore, attempts to increase plasma t-PA levels by the use of retinoids should be directed at finding RARß-specific ligands with a higher transactivation capacity than that of RA, rather than at increasing plasma RA levels.

Taken together, our results provide evidence that the induction of t-PA synthesis by RA in HUVECs occurs via a 2-step process in which one receptor subtype, RARα, is required for the induction of a second subtype, RARß2, which subsequently mediates t-PA synthesis. These apparently subtype-specific functions of RARs in HUVECs are accompanied by a different ligand dependency of each step; whereas RA and 9-*cis* are equipotent in inducing RARß, RA shows a much higher efficacy than 9-*cis* RA in inducing t-PA synthesis in cells with elevated RARß levels.

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CHAPTER 4

DIFFERENCES IN METABOLISM AND ISOMERIZATION OF ALL-*TRANS* RETINOIC ACID AND 9-*CIS* RETINOIC ACID BETWEEN HUMAN ENDOTHELIAL CELLS AND HEPATOCYTES

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SUMMARY

Retinoic acid stimulates the expression of tissue-type plasminogen activator (t-PA) in vascular endothelial cells *in vitro* and enhances t-PA levels in plasma and tissues *in vivo*. Compared with the *in vivo* situation, high retinoic acid concentrations are required to induce optimally t-PA expression *in vitro*. These findings led us to study retinoic acid metabolism in cultured human endothelial cells. For comparison, these studies were also performed in the human hepatoma cell line, HepG2, and key experiments were repeated with human primary hepatocytes. Both hepatocyte cultures gave very similar results. Human endothelial cells were shown to possess an active retinoic acid metabolizing capacity, which is quantitatively comparable to that of hepatocytes, but different from that of hepatocytes in several qualitative aspects. Our results demonstrate that all-*trans*-retinoic acid is quickly metabolized by both endothelial cells and hepatocytes. All-*trans*-retinoic acid induces its own metabolism in endothelial cells but not in hepatocytes. 9-*cis*-Retinoic acid is degraded slowly by endothelial cells, whereas hepatocytes metabolize 9-*cis*-retinoic acid very quickly. Furthermore, our data show that hepatocytes, but not endothelial cells, detectably isomerise all-*trans*-retinoic acid to 9-*cis*-retinoic acid and vice versa. In both endothelial cells and hepatocytes all-*trans*-retinoic acid metabolism was inhibitable by the cytochrome P-450 inhibitors liarozole (10 μM) and ketoconazole (10 μM), albeit to different extents and with different specificities. In the presence of the most potent retinoic acid-metabolism inhibitor in endothelial cells, liarozole, at least 10-fold lower all-*trans*- retinoic acid concentrations were required than in the absence of the inhibitor to obtain the same induction of t-PA. In conclusion, our results clearly demonstrate that all-*trans*-retinoic acid and 9-*cis*-retinoic acid are actively but differently metabolized and isomerised by human endothelial cells and hepatocytes. The rapid metabolism of retinoic acid explains the relatively high concentrations of retinoic acid required to induce t-PA in cultured endothelial cells.

INTRODUCTION

Retinoic acid is an important physiological regulator of plasma levels of tissue-type plasminogen activator (t-PA), a crucial enzyme in fibrin-clot surveillance and in maintaining vascular patency [1-4]. Analysis of t-PA levels in the plasma and tissues of rats undergoing retinoic acid treatment showed an increase in t-PA levels by 50% [3,4]. However, plasma t-PA levels in vitamin A-deficient rats were found to be about three-fold lower than control values [4], and retinoic acid treatment of these animals restored plasma t-PA activity to control levels (M. Lansink, unpublished results). These *in vivo* findings also illustrate that under normal physiological conditions retinoic acid levels in plasma and tissues are sufficient to maintain almost maximal retinoic acid-dependent t-PA expression.

t-PA in the circulation originates mainly from the vascular endothelium [5]. We and others have demonstrated that the t-PA stimulating effect of retinoic acid can be mimicked in cultured human endothelial cells [3,6,7]. The induction of t-PA by retinoic acid in endothelial cells was shown to be mediated by retinoic acid receptors (RAR) [8-10]. In line with this receptor-mediated process, we found retinol (vitamin A) to be only a weak inducer of t-PA synthesis [3].

During our studies on t-PA regulation by retinoic acid in cultured human endothelial cells two outstanding questions emerged. Firstly, in our *in vitro* experiments 1–10 μM retinoic acid is required to induce optimally t-PA synthesis [8], whereas *in vivo* plasma retinoic acid levels between 3.6 nM and 6.3 nM [11,12] apparently suffice to maintain nearly maximal t-PA expression. The high retinoic acid concentrations necessary in endothelial cell cultures might be due to a rapid metabolism of retinoic acid by these cells, but this has not been documented before. Considering the major endothelial surface area, 720 m^2 [13] or 1500 m^2 [14] in adults, an active metabolism of retinoic acid by these cells would suggest that a substantial part of retinoic acid degradation in the body may occur in the endothelium. Secondly, we found that *in vitro* both all-*trans*-retinoic acid and 9-*cis*-retinoic acid are equipotent in stimulating t-PA synthesis in cultured human endothelial cells [8]. However, *in vivo* plasma levels of 9-*cis*-retinoic acid are much lower than those of all-*trans*-retinoic acid: in humans 9-*cis*-retinoic acid concentrations are below the detection limit of 1 nM [15]. However, in some tissues like mouse liver and kidney 9-*cis*-retinoic acid indeed has been shown to be present at significant levels [16]. This raises the question of whether 9-*cis*-retinoic acid also accumulates in the endothelium, either by uptake from the plasma or by isomerization from all-*trans*-retinoic acid, and thus contributes to the regulation of t-PA *in vivo*. To address these questions we have studied the uptake and metabolism of all-*trans*-retinoic acid and 9-*cis*-retinoic acid in cultured human endothelial cells. For comparison, we performed these experiments also in HepG2 cells and human hepatocytes, which are recognized sites for retinoic acid metabolism [17-19].

MATERIALS AND METHODS

Materials

All-*trans*-[^{3}H]retinoic acid (50.3–53.9 Ci/mmol) was obtained from DuPont NEN. All-*trans*-retinoic acid, dimethylsulfoxide (Me_2SO), ketoconazole and metyrapone were purchased from Sigma. Liarozole was a kind gift from Dr J.P. van Wauwe, Janssen Research Foundation, Beerse, Belgium. 9-*cis*-Retinoic acid was kindly provided by Drs M. Klaus and C. Apfel (Hoffmann-LaRoche, Basel, Switzerland). Stock solutions of all-*trans*-retinoic acid and 9-*cis*-retinoic acid (10 mM) were prepared in Me_2SO and stored at -20 °C. Stock solutions of test compounds were either diluted with incubation medium to the final test concentrations immediately before the start of an experiment or were added into the incubation medium during an experiment at the time indicated. All experiments involving retinoids were carried out in subdued light and the tubes containing the retinoid solutions were covered with aluminium foil. The enzyme immunoassay kit for determination of human t-PA antigen (Thrombonostika t-PA) was obtained from Organon Teknika. All other materials used have been specified in the methods described or in the relating references.

Cell culture

Endothelial cells were isolated from human umbilical cord veins using the method of Jaffe *et al.* [20]. The cells were cultured in fibronectin-coated 10-cm^2 dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) heat-inactivated newborn calf serum, 10% (by vol.) human serum, 150 µg/ml endothelial cell growth supplement [21], 2 mM L-glutamine, 5 IU/ml heparin, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The medium was replaced every 2-3 days. Subcultures were obtained by trypsin/EDTA treatment of confluent monolayers at a split ratio of 1:3. The cells were used for experiments at first or second passage.

The human hepatoma cell line HepG2 was cultured in uncoated dishes in DMEM, supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells of early passage (<15) were used for our metabolism studies, since cells at higher passage number gradually lost their retinoic acid metabolizing capacity. The procedure and conditions for the isolation and culture of human hepatocytes have been described by Kooistra *et al.*[22].

Experimental conditions for retinoic acid metabolism studies

For retinoic acid metabolism studies, human endothelial cells or HepG2 cells were grown to confluency. In the case of HepG2 cells, some cell aggregates were formed also. Primary cultures of human hepatocytes were used 24 h after seeding. The day before the experiment, the culture medium was replaced by incubation medium, viz DMEM supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) human serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin for endothelial cells; DMEM supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin for HepG2 cells; and DMEM supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) heat-inactivated fetal calf serum, 2 mM L-glutamine, 10 nM insulin, 50 nM dexamethasone, 100 U/ml penicillin and 100 µg/ml streptomycin, the standard culture medium for primary human hepatocyte cultures [22]. At the start of an experiment, fresh incubation medium containing the appropriate test compound or vehicle was added. In experiments with the cytochrome P-450 inhibitors liarozole, ketoconazole and metyrapone, the inhibitors were always present 1 h before the addition of retinoic acid. Data for the t=0 time points were obtained by collecting media immediately after addition of retinoic acid to the cells. For the other time points the media were collected at the indicated time. Subsequently the cells were washed twice with 1 ml ice-cold NaCl/P_i (0.15 M NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). The medium and both wash steps were combined. Cells were collected by scraping with a rubber policeman in 1 ml NaCl/P_i. Cells and media were immediately frozen in liquid nitrogen and stored at -80 °C until extraction.

Extraction of retinoic acid metabolites from media and cells

All extraction and analytical procedures were carried out in a darkened room using brown glass tubes to protect the retinoids from exposure to light. An internal standard consisting of a known amount of TIMOTA [all-*trans*-7-(1,1,3,3-tetramethyl-5-indanyl)-3-methyl-octa-2,4,6-trienoic acid], kindly provided by Dr A. Levin (Hoffmann-LaRoche Inc, Nutley, NJ), was added in 100 µl of ethanol to each sample in order to monitor the recovery of retinoic acid during the extraction and HPLC procedures [23]. Retinoids were extracted from the media and cells using a modification of the procedure described by Tang and Russell [12,24]. Briefly, the media and cell suspensions were extracted with 8-12 and 6 ml chloroform/methanol (2:1, by vol.), respectively. Next, the chloroform extracts were concentrated by evaporation under a gentle stream of N_2 to a final volume of about 1 ml. This retinoid-containing chloroform extract was then applied to a 500 mg aminopropyl solid-phase extraction column (Baxter Labs Inc.) that had previously been equilibrated with hexane. Neutral lipids present in the extract were eluted with 1 column volume chloroform/isopropanol (2:1, by vol.) and discarded. Subsequently, the retinoids were eluted from the aminopropyl column with 2.5 column volumes of 2% (by vol.) acetic acid in diethyl ether. The acetic acid/diethyl ether eluates were collected, evaporated to dryness under a gentle stream of N_2, and redissolved in HPLC mobile phase (hexane/acetonitrile/acetic acid, 99.5:0.4:0.1, by vol.) for injection onto the HPLC column.

HPLC analysis

Retinoid levels were determined by normal-phase HPLC employing two silica columns linked in tandem. The silica columns consisted of a 3.9mm x 150 mm Waters 5 µ Resolve (Waters Associates) and a 4.6 mm x 150

mm 3 μ Supelcosil LC-SI (Supelco Inc). The first column was preceded by a Waters silica Guard-PAK guard column. For chromatography, we employed an isocratic system where the mobile phase consisted of hexane/acetonitrile/acetic acid (99.5:0.4:0.1, by vol) flowing at 1.8 ml/min. The mobile phase was made fresh daily and filtered and degassed immediately prior to use. The solvent was delivered by a Varian Star 9010 pump (Varian Instruments). We routinely injected 90 μl sample onto the columns using a Varian Star 9095 Autosampler. Retinoic acid mass was detected at 350 nm using a Spectra-Physics Spectra 100 variable wavelength detector (Spectra Physics Inc., Piscataway, NJ). All-*trans*-[^{3}H]retinoic acid was detected and quantitated with an in-line Berthold LB506C-1 radioactivity monitor (EG&G Berthold, Nashua, NH). All data shown are expressed as percentages of the amount all-*trans* retinoic acid or 9-*cis* retinoic acid present at time zero in media and cells and are corrected for extraction efficiency, as assessed by the recovery of the internal standard TIMOTA. Mean (± SD) recoveries upon extraction were 82.8 ± 4.4 (n=9) and 88.4 ± 2.3 (n=6) for the media and cells, respectively. All data shown are for confluent cells on 10 cm^2 dishes incubated with 1 ml incubation medium. The amounts of cells in our experiments were determined to be about 0.7 x 10^6 for endothelial cells, 2.0 x 10^6 for HepG2 cells and 0.8 x 10^6 for primary hepatocytes on 10 cm^2 . The metabolism studies were usually performed with 1 μM all-*trans*-retinoic acid and amounts of retinoic acid were determined from absorbance profiles (wavelength=350 nm) by comparing integrated peak areas with a standard curve relating peak areas to known masses of standard all-*trans*-retinoic acid. For some experiments, trace amounts (20 nM) all-*trans*- [^{3}H]retinoic acid were added to the 1 μM all-*trans*-retinoic acid; the rate of disappearance of the radiolabeled all-*trans*-retinoic acid in cells and media was always identical to that of the unlabeled all-*trans* retinoic acid. Since determining the amount of retinoic acid by measuring radiaoactivity is more sensitive than by measuring mass, metabolism studies with low retinoic acid concentrations (20 nM) were performed with all-*trans*-[^{3}H]retinoic acid. Half-lives of retinoids were calculated with regression analysis (Slide Write Plus, version 5.01, Advanced Graphichs Software Inc.).

t-PA synthesis experiments

For t-PA synthesis experiments, confluent cultures of human endothelial cells were used at first or second passage, and cells were always refed with incubation medium the day before the experiment, viz DMEM supplemented with 20 mM Hepes, pH 7.4, 10% (by vol.) human serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Conditioned media were obtained by incubating cells at 37 °C for 24 h with fresh incubation medium containing the appropriate concentration of all-*trans*-retinoic acid, 10 μM liarozole or stock solvent Me_2SO (final concentration 0.1%, by vol.). Liarozole was always present 1 h before the addition of retinoic acid to the cells. Conditioned media were centrifuged for 5 min at 8000 rpm in a Microfuge centrifuge to remove cells and cellular debris and were stored at -20 °C until use for t-PA antigen determinations.

RESULTS

Metabolism of all-*trans*-retinoic acid and 9-*cis*-retinoic acid in human endothelial cells and hepatocytes

Cultured human endothelial cells and HepG2 cells were incubated with 1 μM all-*trans*-retinoic acid or 9-*cis*-retinoic acid, and after different time periods the amount of retinoic acid present in the media and cells was determined by HPLC analysis. As shown in Fig. 1A for endothelial cells, all-*trans*-retinoic acid rapidly disappeared from the medium, but only a minor part of it was recovered from the cells; the amount of all-*trans*-retinoic acid associated with the cells reached maximally 2.0% of the added amount of all-*trans*-retinoic acid after 1 h of incubation and then rapidly decreased to levels below the detection limit (2.6 pmol) after 12 h of incubation. The overall disappearance of all-*trans*-retinoic acid from the system (medium and cells together), showed a biphasic pattern (Fig. 1A): an initial phase of about 8 h with a half-life of all-*trans*-retinoic acid of approximately 8 h and a second phase during the

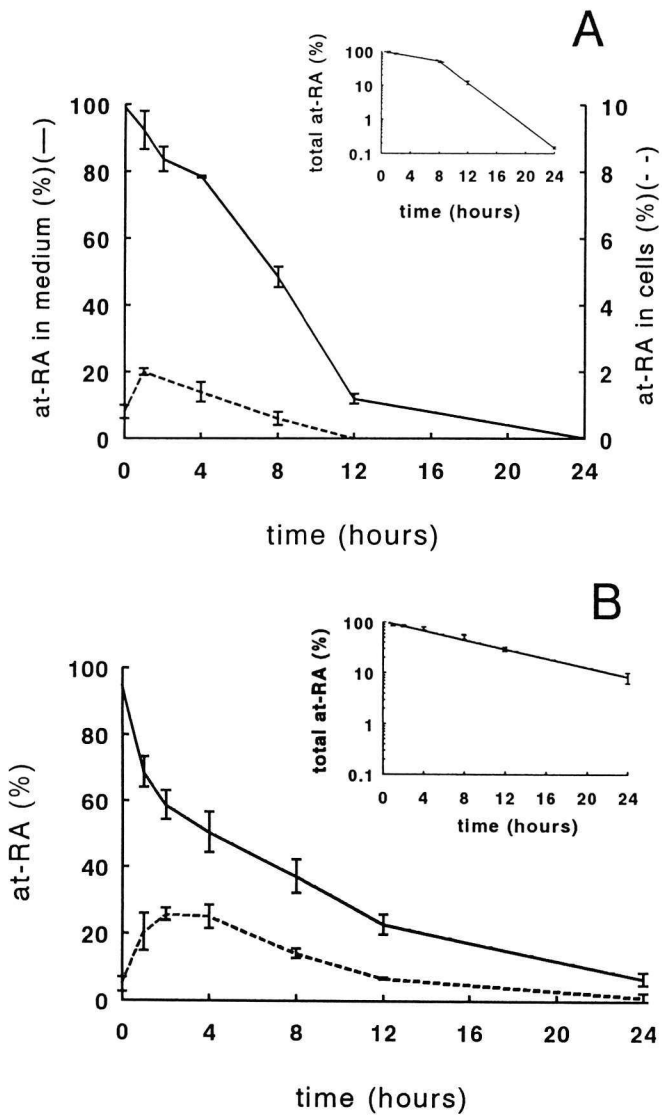

Fig. 1. Disappearance profile of all-*trans*-retinoic acid from the medium (solid line) and cell-associated all-*trans*-retinoic acid (dotted line) in time in cultured human endothelial cells (A) or HepG2 cells (B). In the insert a semilogarithmic plot of the total amount of all-*trans*-retinoic acid (at-RA) present in media + cells as a function of time is shown. Cells were incubated with 1 μM all-*trans*-retinoic acid and at the indicated times cells and media were harvested and analyzed for retinoic acid levels as described in the methods section. The data are expressed as percentages relative to the amount of all-*trans*-retinoic acid added at time point zero. Values shown are means ± SE of three separate experiments.

next 16 h with a half-life of all-*trans*-retinoic acid of approximately 2 h. This biphasic elimination pattern could reflect saturation kinetics, induction of retinoic acid metabolizing capacity or both. Evidence for saturation kinetics comes from retinoic acid elimination studies at a concentration of 20 nM all-*trans*-retinoic acid, where all-*trans*-retinoic acid showed a rapid monophasic disappearance with a half-life of 1.4 h (Fig. 7A). To examine whether all-*trans*-retinoic acid at a concentration of 1 μM is capable of inducing retinoic acid

metabolizing capacity, we incubated cells for 24 h with or without 1 μM all-*trans*-retinoic acid, and then determined the catabolism of 20 nM all-*trans*-[^{3}H]retinoic acid 4 h later. Whereas under control conditions 36 ± 12 % (n=3) of the all-*trans*-[^{3}H]retinoic acid was left in the medium, the medium of cells pretreated with 1 μM all-*trans*-retinoic acid contained only 12 ± 4% (n=3) of the added all-*trans*-[^{3}H]retinoic acid. These results indicate that both saturation kinetics and induced retinoic acid catabolism play a role in the elimination of 1 μM all-*trans*-retinoic acid. All-*trans*-retinoic acid was relatively stable in the absence of cells: at the end of a 24 h incubation of 1 μM all-*trans*-retinoic acid in incubation medium at 37 °C, 90 ± 10% (n=6) of all-*trans*-retinoic acid was still intact. Together these results demonstrate that cultured human endothelial cells contain an active all-*trans*-retinoic acid metabolizing capacity, which is increased by incubating the cells in the presence of 1 μM all-*trans*-retinoic acid. The data also suggest that the metabolizing capacity of endothelial cells is a limiting factor in the rate at which all-*trans*-retinoic acid is metabolized.

The overall disappearance rate of 1 μM all-*trans*-retinoic acid in HepG2 incubations is comparable to that during the initial phase of 1 μM all-*trans*-retinoic acid metabolism in endothelial cells, and showed a half-life of about 7 h (Fig. 1B). However, in contrast to endothelial cells, HepG2 cells showed no increase in all-*trans*-retinoic acid disappearance rate upon prolonged incubation (Fig. 1B), and no difference in disappearance rate was observed for 1 μM and 20 nM all-*trans*-retinoic acid (Fig. 7B). In comparison to endothelial cells, HepG2 cells contained a high cell-associated all-*trans*-retinoic acid fraction, reaching maximally 25.7% of the added amount of all-*trans*-retinoic acid after 2 h of incubation (Fig. 1B). This reflects, at least partly, the larger cell volume and cell numbers of HepG2.

9-*cis*-Retinoic acid (1 μM) is metabolized only slowly by cultured human endothelial cells showing a half-life of about 37 h over the entire incubation period of 24 h (Fig. 2A). No specific accumulation of 9-*cis*-retinoic acid in the endothelial cells was observed: the amount of 9-*cis*-retinoic acid recovered from the cells varied between maximally 1.8% at 2 h and 1.2% at 24 h (Fig. 2A). In contrast to endothelial cells, HepG2 cells metabolize 9-*cis*- retinoic acid fast with a half-life of 3.4 h and at a higher rate than all-*trans*-retinoic acid (Fig. 2B). The amount of HepG2 cell-associated 9-*cis*-retinoic acid reached maximally 8.1% after 1 h, and then rapidly declined to 0.1% after 24 h. The lower levels of 9-*cis*-retinoic acid relative to all-*trans*-retinoic acid recovered from HepG2 cells may be explained by more rapid metabolism of 9-*cis*-retinoic acid and its conversion to all-*trans*-retinoic acid.

For a small number of experiments, the metabolism of all-*trans*-retinoic acid and 9-*cis*-retinoic acid was examined in primary cultures of human hepatocytes. All-*trans*-retinoic acid (1 μM) and 9-*cis*-retinoic acid (1 μM) were metabolized by primary hepatocytes at a similar rate to that found with HepG2 cells, with 9-*cis*-retinoic acid being metabolized at a higher rate than all-*trans*-retinoic acid (Fig. 3).

Similar to HepG2 cells, the maximal amount of cell-associated all-*trans*-retinoic acid (9.6% ± 0.2, *n*=2) was higher than that of 9-*cis*-retinoic acid (3.9% ± 0.4, *n*=2) 2 h after addition of the respective retinoic acid isoforms. These results provide confidence that the studies of retinoic acid metabolism in HepG2 cells are physiologically relevant.

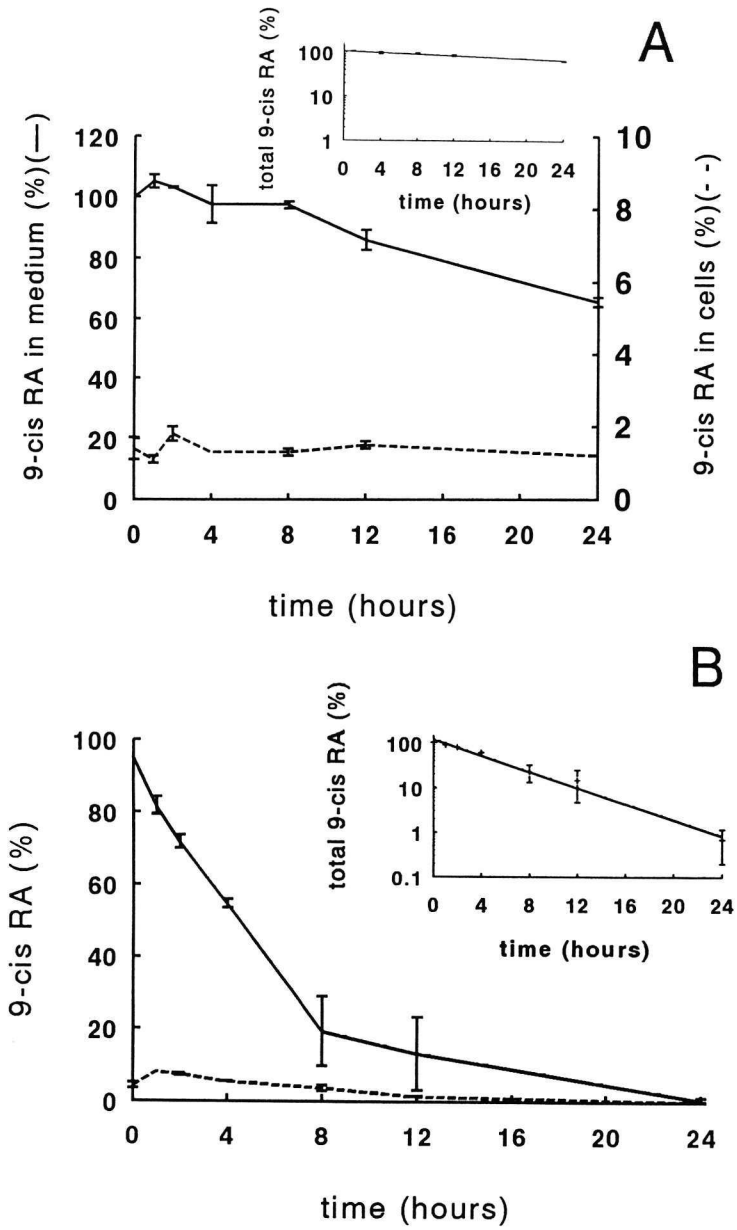

Fig.2. Disappearance profile of 9-*cis*-retinoic acid from the medium (solid line) and cell-associated 9-*cis*-retinoic acid (dotted line) in time in cultured human endothelial cells (A) or HepG2 cells (B). In the insert a semilogarithmic plot of the total amount of 9-*cis*-retinoic acid (9-cis RA) present in media + cells as a function of time is shown. Cells were incubated with 1 μM 9-*cis*-retinoic acid and at the indicated times cells and media were harvested and analyzed for 9-*cis*-retinoic acid levels as described in Materials and Methods. The data are expressed relative to the amount of 9-*cis* retinoic-acid added at time point zero. Values shown are means ± SE of three separate experiments.

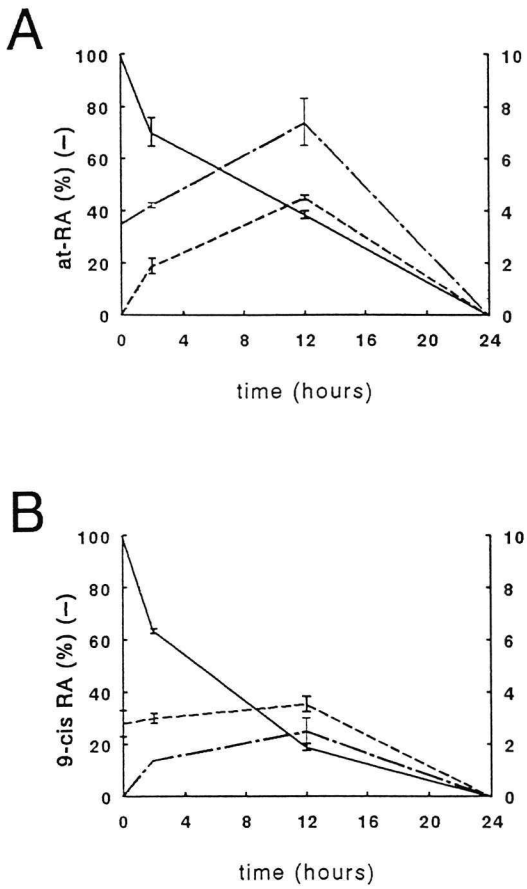

Fig.3. Disappearance profiles. (A) Disappearance profile of all-*trans*-retinoic acid (at-RA) from the medium of primary human hepatocytes cultures (solid line) and formation of 9-*cis*-retinoic acid (9-cis RA) and 13-*cis*-retinoic acid (13-cis RA). Cells were incubated with 1 μM all-*trans*-retinoic acid and at the indicated times media were harvested and analyzed for all-*trans*-retinoic acid, 9-*cis*-retinoic acid and 13-*cis*-retinoic acid levels as described in the Materials and Methods. The data are expressed as percentages relative to the amount of all-*trans*-retinoic acid added at time point zero. Values shown are means ± ranges of two separate experiments. (B) Disappearance profile of 9-*cis*-retinoic acid from the medium of primary human hepatocytes cultures (solid line) and formation of all-*trans*-retinoic acid and 13-*cis*-retinoic acid. Cells were incubated with 1 μM 9-*cis*-retinoic acid and at the indicated times media were harvested and analyzed for 9-*cis*-retinoic acid, all-*trans*-retinoic acid and 13-*cis*-retinoic acid levels as described in the Material and Methods. The data are expressed as percentages relative to the amount of 9-*cis* retinoic acid added at time point zero. Values shown are means ± ranges of two separate experiments.

Isomerization of all-*trans*-retinoic acid and 9-*cis*-retinoic acid in human endothelial cells and hepatocytes

To evaluate whether human endothelial cells are able to form 9-*cis*-retinoic acid from all-*trans*-retinoic acid, the amount of 9-*cis*-retinoic acid (and 13-*cis*-retinoic acid, another isomer of all-*trans*-retinoic acid) in the medium and cells was determined at different time intervals after the addition of all-*trans*-retinoic acid (for a characteristic HPLC profile of medium see Fig 4A). The total amounts of 9-*cis*-retinoic acid and 13-*cis*-retinoic acid recovered from the endothelial cells and medium together did not exceed the spontaneously formed amounts of 9-*cis*-retinoic acid and 13-*cis*-retinoic acid in medium incubated without cells, and accounted for 2.9% ± 1.2 and 4.8 ± 1.5% (n=6), respectively after 24 h incubation at 37 °C. Similarly, no cell-dependent isomerization of 9-*cis*-retinoic acid to all-*trans*-retinoic acid was observed (data not shown). After 24 h incubation of medium containing 1 μM 9-*cis*-retinoic acid under

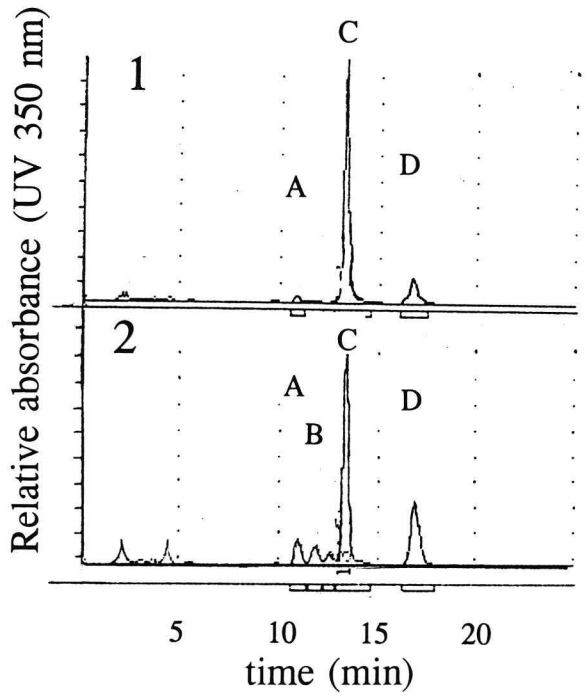

Fig.4. HPLC chromatogram of extract prepared from endothelium medium (panel 1) and HepG2 medium (panel 2), 8 hours after addition of 1 μM all-*trans*-retinoic acid and 20 nM all-*trans*-[^{3}H]retinoic acid to the cells. The chromatogram was obtained by detection of the absorbance at 350 nm. Peak A, 13-*cis*-retinoic acid; peak B, 9-*cis*-retinoic acid; peak C, all-*trans*-retinoic acid; peak D, TIMOTA. All-*trans*-retinoic acid, 13-*cis*-retinoic acid and 9-*cis*-retinoic acid were identified by co-elution with known amounts of respective standard solutions.

cell-free conditions at 37 °C, 4.4% ± 0.5% (n=3) all-*trans*-retinoic acid was formed, whereas the amount of 13-*cis*- retinoic acid was below detection limits (n=3).

In contrast to endothelial cells, when all-*trans*-retinoic acid was added to HepG2 cell cultures, both 9-*cis*-retinoic acid and 13-*cis*-retinoic acid were found to accumulate in both cells and media to levels which were significantly greater than those observed when all-*trans*-retinoic acid was added to media with no cells present (see Fig. 4B for a characteristic HPLC profile for the medium). The amounts of 9-*cis*-retinoic acid and 13-*cis*-retinoic acid (expressed as percentage of the amount of all-*trans*-retinoic acid added at t=0) in the media and cells at different time points after addition of all-*trans*-retinoic acid are shown in Fig 5. In the HepG2 cells the amount of 9-*cis*-retinoic acid was maximally 1.2% after 1 h, remained high until 12 h and then started to decline (Fig. 5A). The formed 9-*cis*-retinoic acid also diffused from the cells into the medium, and reached maximally a level of 4.7 % at 8 h. In addition to the formation of 9-*cis*-retinoic acid, HepG2 cells also generated 13-*cis*-retinoic acid (Fig. 5B). Starting at 4% 13-*cis*-retinoic acid at time zero, the amount of 13-*cis*-retinoic acid in the medium increased to 5.4% at 8 h, and then slowly decreased to 2.1% at 24 h. The amount of 13-*cis*-retinoic acid in the cells peaked at 2.9 % after 2 h and subsequently fell to 0.6% at 24 h.

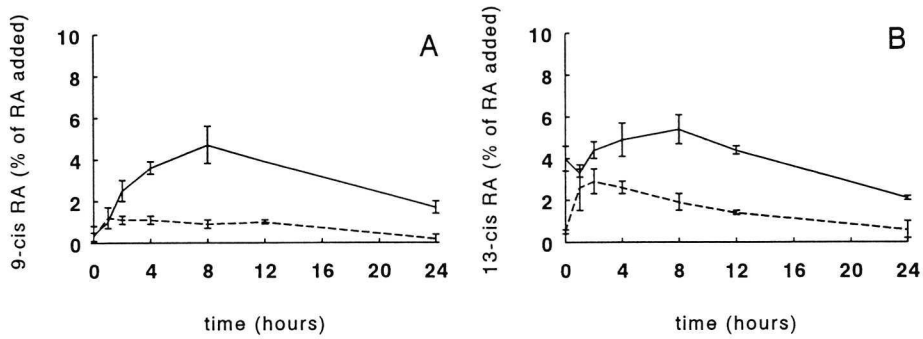

Fig.5. Formation of 9-*cis*-retinoic acid (A) and 13-*cis*-retinoic acid (B) from all-*trans* retinoic-acid by hepatocytes. HepG2 cells were incubated with 1 μM all-*trans*-retinoic acid and after different time points the retinoic acid isomers were determined in the media (solid lines) and in the cells (dotted lines). The total amount all-*trans*-retinoic acid at time zero in media and cells was set 100% and the data are expressed relative to this. The values represent the average (± SE) of three determinations.

When 9-*cis*-retinoic acid was added to HepG2 cell cultures, both all-*trans*-retinoic acid and 13-*cis*-retinoic acid were found to accumulate, in a time-dependent manner, in cells and media (Fig. 6). A maximal level of 5.4% all-*trans*-retinoic acid (of the amount of 9-*cis*-retinoic acid added at *t*=0) in the cells was reached 4 h after addition of 9-*cis*-retinoic acid and in the medium a maximum amount of 8.9% was present at 4 h (Fig. 6A). Although at lower levels than all-*trans*-retinoic acid, 13-*cis*-retinoic acid could also be detected in the cells and in the media (Fig. 6B).

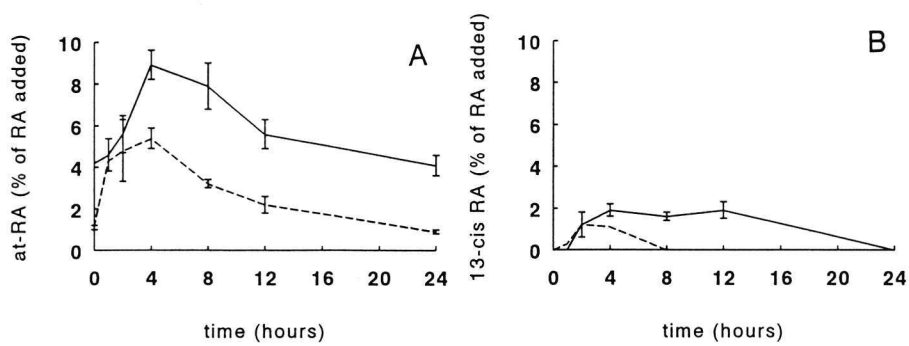

Fig.6. Formation of all-*trans*-retinoic acid (A) and 13-*cis*-retinoic acid (B) from 9-*cis*-retinoic acid by hepatocytes. HepG2 cells were incubated with 1 μM 9-*cis*-retinoic acid and at different time points the amounts of retinoic acid isomers were determined in the media (solid lines) and in the cells (dotted lines). The total amount of 9-*cis*-retinoic acid at time zero in media and cells was set 100% and the data are expressed relative to this. The values represent the average (± SE) of three determinations.

Comparison of the data in Figures 5 and 6 shows that a greater conversion of 9-*cis* retinoic acid to all-*trans* retinoic acid occurs, than vice versa. This is consistent with what would be expected thermodynamically, since at equilibrium only a small part of retinoic acid (± 15%) is in the 9-*cis*-isomeric form [25]. The isomerisation of both all-*trans*-retinoic acid and 9-*cis*-retinoic acid by primary hepatocytes resembled that by HepG2 cells as shown in Fig. 3.

Inhibition of retinoic acid metabolism by human endothelial cells and HepG2 cells by inhibitors of cytochrome P-450

To assess whether the metabolism of all-*trans*-retinoic acid occurs via a cytochrome P-450 (P-450)-dependent mechanism we studied the effect of various P-450 inhibitors on the degradation of 20 nM all-*trans*-[^{3}H]retinoic acid in human endothelial cells. We have used liarozole and ketoconazole, which have been shown to inhibit retinoic acid metabolism in rats *in vivo*, and to block 4-hydroxylation of retinoic acid *in vitro* [26, 27], and metyrapone, which is a more general P-450 inhibitor [28]. We chose to perform these metabolic studies with 20 nM all-*trans*-retinoic acid, because at this concentration retinoic acid shows a monophasic disappearance profile. Liarozole at a concentration of 10 μM is a very potent inhibitor of all-*trans*-retinoic acid degradation in endothelial cells, and increases the half-life from 1.4 h to 17.2 h (Fig. 7A). In the presence of 10 μM ketoconazole, the half-life of all-*trans*-retinoic acid was 4.5 h, whereas with 10 μM metyrapone no inhibition of all-*trans*-retinoic acid metabolism was seen. Liarozole (10 μM) was also a very effective inhibitor of 9-*cis*-retinoic acid metabolism; in the presence of liarozole 94% 9-*cis*-retinoic acid was left after 24 h incubation (data not shown) compared with 65% in the absence of the inhibitor.

Different from endothelial cells, in HepG2 cells liarozole and ketoconazole were about equipotent in inhibiting all-*trans*-retinoic acid metabolism (Fig. 7B), and the half-lives of total all-*trans*-retinoic acid were increased only about threefold, from 7.8 h under control conditions to about 22.7 h and 18.9 h by 10 μM liarozole and 10 μM ketoconazole, respectively. Again, 10 μM metyrapone was a very weak inhibitor of all-*trans*-retinoic acid metabolism by HepG2 cells resulting in a half-life of 9 h. The metabolism of 9-*cis*-retinoic acid was not inhibited by liarozole (data not shown). These results show that all-*trans*-retinoic acid and 9-*cis*-retinoic acid metabolism in human endothelial cells and HepG2 cells can be inhibited by specific cytochrome P-450 inhibitors. The different efficacy of the P-450 inhibitors in the two cell types suggest that different P-450 enzymes may be involved in retinoic acid metabolism in endothelial cells and HepG2 cells.

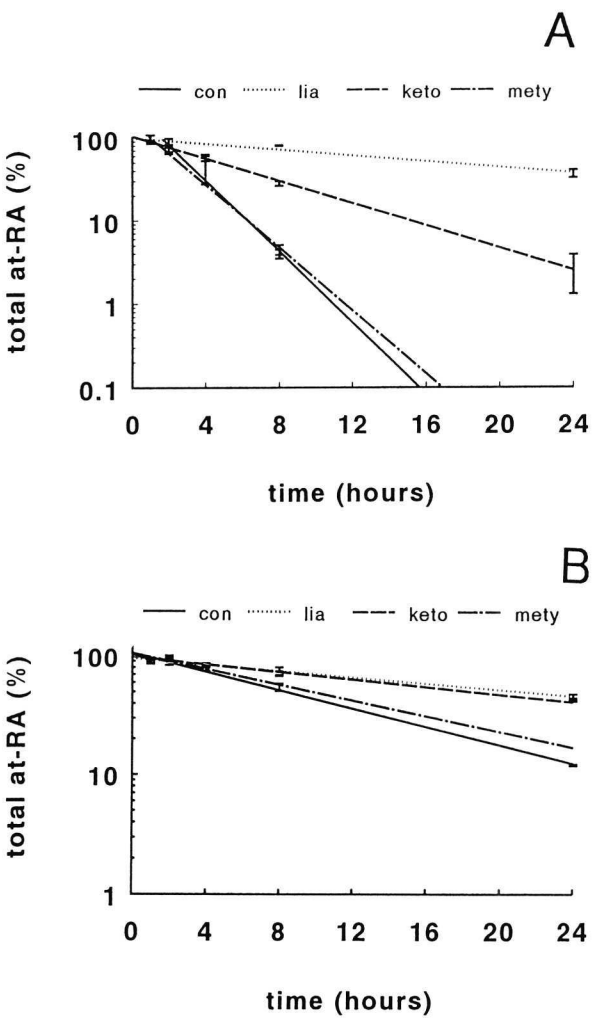

Fig.7. Effects of liarozole (lia), ketoconazole (keto), metyrapone (mety) and vehicle (con), 0.1% (by vol.) Me_2SO, on the metabolism of all-*trans*-retinoic acid (at-RA) by cultured human endothelial cells (A) and HepG2 (B) cells. Cells were incubated with 20 nM all-*trans*- [^{3}H]retinoic acid and after the indicated times media and cells were collected and analyzed as described. The inhibitors were added 1 h before incubation with all-*trans*-[^{3}H]retinoic acid. The disappearance of total all-*trans*-retinoic acid from media and cells is plotted on a semilogarithmic scale. The values shown represent the average of two incubations with ranges indicated by error bars.

Effects of P-450 inhibitors on the induction of t-PA by retinoic acid

To test the influence of all-*trans*-retinoic acid metabolism on t-PA induction, endothelial cells were incubated with different concentrations of all-*trans*-retinoic acid in the presence or absence of liarozole and t-PA production was determined. In the presence of liarozole, at least 10-fold lower all-*trans*-retinoic acid concentrations were required than in the absence of the inhibitor to obtain the same induction of t-PA (Fig. 8).

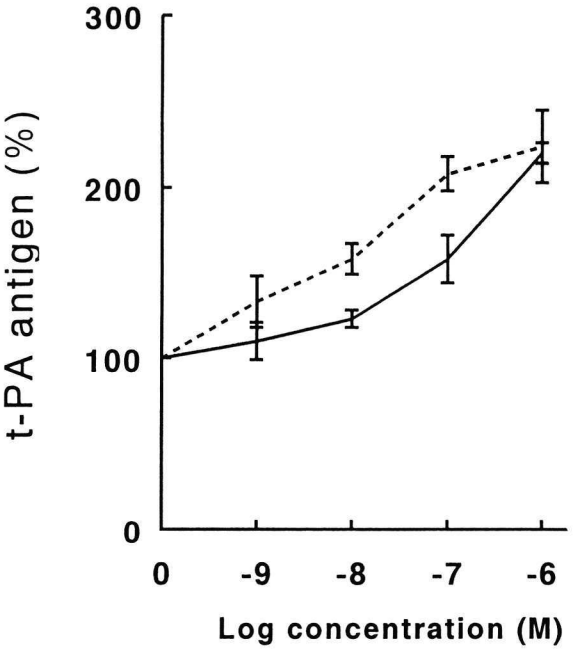

Fig. 8. Effects of all-*trans*-retinoic acid alone (solid line) or in combination with liarozole (dotted line) on t-PA synthesis by cultured human endothelial cells. The cells were incubated for 24 h with differing concentrations of all-*trans*-retinoic acid alone or in combination with 10 μM liarozole, and conditioned media were analyzed for t-PA antigen. The data are expressed as percentages relative to the control value. The data shown are from one representative experiment of three performed and data are expressed as mean values of duplicate determinations with ranges indicated by error bars.

DISCUSSION

Circulating t-PA plays a crucial role in fibrin-clot surveillance and a long-term goal of our research has been to establish whether manipulation of plasma retinoic acid levels can serve as an effective means for elevating blood t-PA levels and consequently lessen the risk for thrombus formation. To this end in previous work, we and others have shown that retinoic acid is a regulator of t-PA gene expression [3,6,7] and that t-PA levels in the plasma and tissues of rats depend on their retinoic acid status [3,4]. In cell culture experiments, we showed that media supplemented with all-*trans*-retinoic acid concentrations ranging between 1 μM and 10 μM are required to optimally induce t-PA expression in human endothelial cells [8], whereas *in vivo* plasma all-*trans*-retinoic acid levels between 3.6 nM and 6.3 nM [11,12] apparently suffice to maintain high t-PA expression. This discrepancy between the *in vitro* and *in vivo* concentrations of retinoic acid required to induce t-PA expression suggested to us that *in vitro* in vascular endothelial cells retinoic acid metabolism might be an important process which can modulate cellular responses towards retinoic acid. To gain understanding of this possibility, we carried out studies of all-*trans*-retinoic acid and 9-*cis*-retinoic acid uptake and metabolism in primary cultures of human endothelial cells. To link our studies to other cell types to which circulating retinoic acid will have extensive exposure and which are

thought to be importantly involved in retinoic acid metabolism [29], we carried out parallel studies in cultured primary human hepatocytes and the human HepG2 hepatocyte cell line.

Four major findings emerged from our studies of retinoic acid uptake and metabolism by human endothelial cells and hepatocytes. First, all-*trans*-retinoic acid is taken up and rapidly metabolized by both human endothelial cells and hepatocytes. However, metabolism of all-*trans*-retinoic acid was found to be inducible in endothelial cells which had been pre-exposed to all-*trans*-retinoic acid. This induction of all-*trans*-retinoic acid metabolism was not observed in hepatocytes. Secondly, the uptake and rate of metabolism of 9-*cis*-retinoic acid in endothelial cells was markedly different from that of all-*trans*-retinoic acid. Moreover, the rate of uptake and metabolism of 9-*cis*-retinoic acid by hepatocytes was very different from that of endothelial cells. Third, through the use of a sensitive normal-phase HPLC procedure for measuring all-*trans*-retinoic acid and 9-*cis*-retinoic acid levels in the cultured cells and corresponding media, we were able to demonstrate that hepatocytes possess the capacity to catalyze the interconversion of all-*trans*-retinoic acid and 9-*cis*-retinoic acid. Endothelial cells did not catalyze either the isomerization of all-*trans*-retinoic acid to its 9-*cis* isomer or the isomerization of 9-*cis*-retinoic acid to the all-*trans* isomer. Finally, metabolism of all-*trans*-retinoic acid and 9-*cis*-retinoic acid by both human endothelial cells and HepG2 cells can be inhibited by exposure of the cells to the cytochrome P-450 inhibitors liarozole and ketoconazole.

A surprising finding of this study is the active role played by cultured human endothelial cells in metabolizing all-*trans* retinoic acid, an activity hitherto unknown. This rapid metabolism of all-*trans*-retinoic acid by cultured human endothelial cells probably explains the relatively high all-trans-retinoic acid levels which are necessary to induce t-PA expression *in vitro* compared with *in vivo*, where a steady-state plasma level of all-*trans*-retinoic acid is present. In the presence of the most potent all-*trans*-retinoic acid metabolism inhibitor in endothelial cells, liarozole, at least 10-fold lower all-*trans*-retinoic acid concentrations were required than in the absence of the inhibitor to obtain the same induction of t-PA. This metabolism by endothelial cells may be an important factor in establishing plasma all-*trans*-retinoic acid levels, considering that the major endothelial surface area in the adult human is estimated to be 720–1500 m^2 [13,14], and is of obvious relevance for the pharmacologic use of all-*trans*-retinoic acid. It should be noted that all-*trans*-retinoic acid is initially a very effective drug for treatment of acute promyelocytic leukemia (APL) but, with time, patients develop a tolerance for the all-*trans*-retinoic acid (for review see [31] and references therein). This tolerance has been attributed to an induction of all-*trans*-retinoic acid metabolism within the patient. It is interesting that for our short-term cell culture studies primary human endothelial cells but not human hepatocytes display an inducible metabolism of all-*trans*-retinoic acid. Recently, White et al. described an inducible retinoic acid 4-hydroxylase, designated P450RAI [30]. It will be of interest to learn whether retinoic acid induces

P450RAI in endothelial cells.

Hepatocytes were found to differ from endothelial cells in their abilty to metabolize retinoic acid in two distinct ways. First, hepatocytes metabolize 9-*cis*-retinoic acid at a rate which is much greater than that observed in endothelial cells. The rate of 9-*cis*-retinoic acid metabolism by hepatocytes is even greater than for all-*trans*-retinoic acid. Second, hepatocytes but not endothelial cells are able to form 9-*cis*-retinoic acid from all-*trans*-retinoic acid, and vice versa. Although our data do not provide us with insight as to whether this process is enzymatic or non-enzymatic in nature, they are consistent with the observation of Urbach and Rando [32] that thiol groups present in heat-inactivated hepatic microsomal membranes are able to bring about the isomerization of all-*trans*-retinoic acid to the 9-*cis* isomer. It would seem from these data and data from investigations of retinoic acid metabolism by human keratinocytes [33] and by human mammary carcinoma cells [34] that the abilities of cells to take up and metabolize retinoic acid are diverse and cell-type dependent.

It is well established that the P-450 system is involved in the metabolism of retinoic acid [35] and that treatment of rats with P-450 inhibitors such as liarozole and ketoconazole bring about increased blood and tissue levels of retinoic acid [27]. Liarozole has been shown to disrupt the metabolism of all-*trans*-retinoic acid at two levels: the conversion of all-*trans*-retinoic acid to all-trans-4-hydroxyretinoic acid (which is then oxidized to all-*trans*-4-keto retinoic acid) and the metabolism of all-*trans*-4-keto retinoic acid [36]. Our studies with P-450 inhibitors indicate the involvement of different metabolic routes and/or p-450 isoenzymes in all-*trans*-retinoic acid and 9-*cis*-retinoic acid metabolism in endothelial cells and HepG2 cells. We found that in endothelial cells liarozole (10 μM) increased the half-life of all-*trans*-retinoic acid 12-fold, ketoconazole (10 μM) three-fold, while metyrapone (10 μM) had no effect. In contrast, in HepG2 cells liarozole and ketoconazole are equipotent in inhibiting all-*trans*-retinoic acid metabolism and increased the half-life of all-*trans*-retinoic acid within the cells by approximately three-fold. The metabolism of 9-*cis*-retinoic acid was almost completely inhibited by liarozole in endothelial cells, whereas in HepG2 cells had no effect on 9-*cis*-retinoic acid metabolism. Experiments in rats have shown liarozole to be a much more potent inhibitor of all-*trans*-retinoic acid metabolism than ketoconazole [27]. Administration of liarozole or ketoconazole enhanced endogenous plasma concentrations of all-*trans*-retinoic acid in rats from mostly undetectable values (less than 0.5 ng/ml) to 2.5 ng/ml and 1.3 ng/ml, respectively [27]. Taken together with our observation that liarozole more markedly influences endothelial cell metabolism of all-*trans*-retinoic acid than hepatocyte metabolism, it further substantiates our finding that extrahepatic tissues including the endothelium may significantly contribute to all-*trans*-retinoic acid metabolism *in vivo*.

We have investigated the uptake and metabolism of all-*trans*-retinoic acid and 9-*cis*-retinoic acid by two human cell types which probably represent the two most abundant, and

consequently the two most frequently encountered cell types to which circulating retinoic acid is exposed in the body. Our data suggest that the vascular endothelium plays a significant role in the metabolism of all-*trans*-retinoic acid within the body. This had not been previously suspected. This observation regarding metabolism of all-*trans*-retinoic acid by endothelial cells also provides insight into, and raises questions about, how the body responds to the pharmacological use of all-*trans*-retinoic acid for treatment of disease. Our data also show that the abilities of cells to take up and to metabolize all-*trans*-retinoic acid and 9-*cis*-retinoic acid are cell-type specific. Finally, our data demonstrate that hepatocytes but not endothelial cells are able to catalyze the isomerization of all-*trans*-retinoic acid and 9-*cis*-retinoic acid and again suggest that the metabolism of retinoic acid is different for different cell types. It seems clear from our studies of retinoic acid uptake and metabolism by cells in culture and from studies in other isolated cell systems by others [34,35,37] that retinoic acid metabolism in the intact organism is very complex. Thus, it would seem that not only is retinoic acid action within cells complex and cell-type specific but also that the metabolism of retinoic acid within cells is similarly complex and cell type-specific.

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CHAPTER 5

INCREASED CLEARANCE EXPLAINS LOWER PLASMA LEVELS OF TISSUE-TYPE PLASMINOGEN ACTIVATOR BY ESTRADIOL. EVIDENCE FOR POTENTLY ENHANCED MANNOSE RECEPTOR EXPRESSION IN MICE.

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ABSTRACT

Several studies have demonstrated an inverse relationship between circulating levels of estrogen and tissue-type plasminogen activator (t-PA). The present study was designed to test the hypothesis that estrogens lower plasma levels of t-PA by increasing its clearance from the bloodstream. Ethinylestradiol treatment resulted in a significant increase in the clearance of endogenous, bradykinin-released t-PA in rats [area under the curve 24.9 ng/ml.min in treated animals vs 31.9 ng/ml.min in controls;$p< 0.05$). The clearance rate of recombinant human t-PA in mice was also significantly increased after ethinylestradiol treatment (0.46 ml/min in treated mice vs 0.32 ml/min in controls;$p<0.01$). Two distinct t-PA clearance mechanisms, which are mediated by the LDL-receptor related protein (LRP) on liver parenchymal cells and by the mannose receptor on mainly liver endothelial cells, have been described. Inhibition of LRP by i.v. injection of RAP (receptor associated protein) as a recombinant fusion protein with *Salmonella japonicum* glutathione S-transferase (GST)(GST-RAP) or by overexpression of RAP in the liver of mice by adenoviral gene transduction significantly retarded t-PA clearance in control (from 0.41 ml/min to 0.25 ml/min, $n=5;p<0.001$) and estradiol-treated mice (from 0.66 ml/min to 0.35 ml/min, $n=5;p<0.005$), but did not eliminate the difference in clearance capacity between the two experimental groups. In contrast, administration of a mannose receptor antagonist, mannan, resulted in indentical clearances : 0.22 ml/min in controls and 0.24 ml/min in estradiol-treated mice. Nothern blot analysis showed a six-fold increase in mannose receptor mRNA expression in the non-parenchymal liver cells of estradiol-treated mice, whereas the parenchymal LRP mRNA levels remained unchanged. These findings were confirmed at the protein level by ligand blotting and Western blotting analysis. Our findings demonstrate that ethinylestradiol treatment results in increased plasma clearance of t-PA via a strong induction of the mannose receptor, and provide an explanation for the inverse relationship between estrogen status and plasma t-PA concentrations.

INTRODUCTION

The fibrinolytic system, which is responsible for the dissolution of fibrin in the circulation, is a complex process that requires precise regulation to ensure that it is neither deficient nor excessive. The physiological importance of the system in humans is demonstrated by associations between impaired fibrinolysis and thrombotic events and between excessive fibrinolysis and bleeding complications. Clinical and epidemiological studies suggest that lowered fibrinolytic activity, resulting in enhanced fibrin deposition, is a significant contributor to the development of atherothrombosis (Lijnen and Collen, 1996).

Tissue-type plasminogen activator (t-PA) is a key enzyme in the fibrinolytic process by

converting the inactive proenzyme plasminogen to plasmin, a broad-spectrum protease which cleaves fibrin. Human t-PA is also of particular pharmacological interest because of its value in the treatment of thromboembolic disorders (Lijnen and Collen, 1995). Regulation of t-PA activity in blood is regulated at several levels, including controlled synthesis and release of t-PA from the vascular endothelium, the presence of the physiological inhibitor plasminogen activator inhibitor type-1 (PAI-1), and rapid hepatic clearance of t-PA (Sprengers and Kluft, 1987; Kuiper et al., 1988; Chandler, 1991; Kooistra et al., 1994). It is well-documented that the synthesis of t-PA (and PAI-1) is influenced by a variety of exogenous factors such as hormones, cytokines, growth factors and vasoactive compounds (for a review, see Kooistra 94 et al., and references therein) . Little is known about the regulation of hepatic clearance capacity. Changes in t-PA clearance rate under various physiological and experimental conditions are usually attributed to the more rapid clearance of t-PA as compared to that of t-PA/PAI-1 complexes (Chandler et al., 1997) and/or to changes in the liver blood flow (van Griensven et al., 1996).

There are several indications of an inverse correlation between estradiol and plasma t-PA levels. First, lower plasma concentrations of t-PA are found in women as compared to men (Siegbahn and Ruusuvaara, 1988; Eliasson et al., 1993; Gebara et al., 1995). Secondly, the use of estrogens by women on oral contraceptives or hormone replacement significantly decreases plasma t-PA levels (Gevers Leuven et al., 1987; Petersen et al., 1993; Gebara et al., 1995). Thirdly, the administration of estradiol (in combination with cyproterone acetate) to male-to-female transsexual subjects markedly reduces circulating t-PA concentrations (Kooistra et al., 1996). *In vitro* studies using cultured human vascular endothelial cells failed to demonstrate a direct effect of estrogens on t-PA synthesis (Kooistra et al., 1990, 1996), suggesting to us the possibility that estradiol lowers circulating t-PA levels by increasing hepatic t-PA clearance. In the present communication, we report that ethinylestradiol treatment enhances the clearance rate of endogenous t-PA in rats and exogenously administered recombinant human t-PA in mice. Two major t-PA receptors exist in liver, the low-density-lipoprotein receptor-related protein (LRP) on predominantly liver parenchymal cells and the mannose receptor mainly on liver endothelial cells (Kuiper et al., 1988; Smedsrød et al., 1990; Otter et al., 1992; Bu et al., 1992; Orth et al., 1994). By using specific receptor antagonists we show that the increased clearance of t-PA in ethinylestradiol-treated mice is mediated via the mannose receptor. The role of the mannose receptor in the clearance of t-PA is further substantiated by demonstrating an estradiol-increased mannose receptor expression at the mRNA and protein level. These results provide an explanation for the inverse relationship between estradiol and plasma t-PA concentrations.

MATERIALS AND METHODS

Reagents

Recombinant human t-PA expressed in Chinese hamster ovary cells was obtained from Genentech (San Francisco, CA). Ethinylestradiol, bradykinin, mannan and peanutoil were purchased from Sigma Chemical Co. (St. Louis, MO). Hypnorm was from Janssen Pharmaceutica (Tilburg, The Netherlands) and midazolam from Roche (Mijdrecht, The Netherlands). The mouse monoclonal human mannose receptor antibodies were prepared in our laboratory (Barrett-Bergshoeff et al., 1997). Enzyme immunoassay kits for determination of human t-PA antigen (Thrombonostika t-PA) were obtained from Organon Teknika (Boxtel, The Netherlands). Materials used in the rat t-PA immunoassay (ELISA) have been described previously (Padro et al., 1990). RAP was produced as a recombinant fusion protein with *Salmonella japonicum* glutathione S-transferase (GST) according to a combination of the methods described by Herz et al (1991) and Warshawsky et al (1993). The RAP-GST plasmid was kindly provided by Dr J. Herz (University of Texas, Dallas, TX). Other materials used have been specified in the methods described or in the relating references.

Preparation of recombinant adenoviral vectors

The recombinant adenoviral vectors expressing either the receptor-associated protein (RAP) gene (Ad-RAP) or the ß-galactosidase gene (Ad-ß-Gal) under the control of CMV promoter were kindly provided by Drs T. Willnow and J. Herz, respectively (Willnow et al., 1994; Herz and Gerard 1993). The generation of these vectors and the propagation and titration of the recombinant adenovirus have been described previously (Jong et al., 1996). Briefly, for *in vivo* adenovirus infection, the virus was subjected to two rounds of purification by CsCl gradient centrifugation followed by extensive dialysis against TD buffer (25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.73 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ (pH 7.45)), at 4°C. After dialysis, mouse serum albumin was added to 0.2% (wt/vol) and glycerol to 10% (vol/vol), and aliquots of the virus stocks were frozen in liquid N_2 and stored at -80°C. Routinely virus titers of the stocks varied from $1\text{-}5x10^{10}$ plaque forming units (PFU)/ml. $3x10^9$ PFU in a total volume of 200 µl (diluted with PBS) were injected into the tail vein of LDL-receptor (LDLR)-deficient mice. t-PA clearance studies were performed at day 5 after virus injection.

Experimental design

Animals

C57 black6/B6 mice and Wistar rats were obtained from Iffa-Credo (L'Arbresle, France). LDLR-deficient mice were purchased from the Jackson Laboratory (Bar Harbor, ME). For experiments, mature male rats (8-12 weeks) and male mice (8-14 weeks) were used. The animals were housed as an experimental group with a 12-h light cycle and free access to drinking water and standard (chow) diet. All experimental procedures were performed in accordance with the Netherlands regulations for experiments with animals.

Estradiol treatment

Rats and mice were injected four times subcutaneously with ethinylestradiol (5 mg/kg bodyweight/day), either during four consecutive days or on day 1, 4, 5 and 6. The LDLR-deficient mice (which were used parallelly for VLDL-clearance studies) received three two-weekly subcutaneous injections of 100 µg ethinylestradiol (dissolved in peanut oil); control animals received peanut oil only. Experiments were always performed one day after the last estradiol injection in case of the short-term treatment or two weeks after the third estradiol injection in case of the long-term treatment of LDLR-deficient mice. No differences in t-PA clearance characteristics were observed for the various estradiol-treatment protocols.

Clearance of bradykinin-released t-PA in rats

Ethinylestradiol or vehicle-treated rats were anaesthetized with intraperitoneal Nembutal (60 mg/kg bodyweight), cannulated, and bradykinin (50 µg/kg bodyweight) was injected as a bolus into the vein of the penis. Blood was collected immediately before and at different time intervals after bradykinin injection through a carotid artery cannula, and citrated plasma was prepared. Rat t-PA antigen concentrations were determined in citrated plasma by ELISA (Padró et al., 1990).

Plasma clearance of exogenous t-PA in mice; effect of inhibitors

Ethinylestradiol or vehicle-treated mice were anaesthetized by injection of Hypnorm (0.6 mg fluanison and 19 µg fentanyl citrate/ 30 g bodyweight) and midazolam (0.2 mg/30 g bodyweight). 15 µg recombinant human t-PA in 200 µl sterile saline was injected into a tail vein. Blood (35-40 µl) was collected immediately after t-PA injection and at different time intervals thereafter, and citrated plasma was prepared. Human t-PA antigen levels were determined in citrated plasma by ELISA (Thrombonostica t-PA).In studies where mannan (5 mg/kg body weight) was administered, competitor was injected 1-3 minutes before administration of t-PA. RAP-GST (40 mg/kg bodyweight) was preinjected 1 min before t-PA injection. In case of Ad-RAP or Ad-ß-Gal infections, LDLR-deficient mice were injected with 3 x 10^9 PFU of virus in a total volume of 200 µl (diluted with PBS) into the tail vein, five days before the t-PA clearance studies. At the end of experiments, livers were rapidly removed and immediately frozen in liquid nitrogen for preparation of total RNA or membrane fragments.

Isolation of total RNA and Northern blot analysis. Frozen liver samples were triturated in liquid nitrogen in a mortar and resuspended at 100 mg/ml in guanidinium thiocyanate/phenol-chloroform extraction buffer following the procedure of Chomczynski and Sacchi (1987). The tissue samples were homogenized mechanically using a motor-driven Potter-Elvehjem homogeniser (10 strokes) at 0 °C. Total RNA was isolated according to the method of Chomczynski and Sacchi and electrophoresed in a 1% (wt/vol) agarose gel under denaturing conditions using 1 M formaldehyde, blotted and hybridized as described previously (Lansink and Kooistra, 1996).

cDNA probes

The following cDNA fragments were used as probes in the hybridization experiments: a 6 kb XhoI-EcoRI fragment of the human LRP cDNA (Herz et al., 1988); a 5.1 kb full length EcoRI fragment of the mouse mannose receptor (Harris et al., 1992) (kindly provided by Dr R. Ezekowitz, Harvard, Boston, MA) and a 1.2 kb Pst 1 fragment of a rat glyceraldehyde-3-phosphate dehyrogenase (GAPDH) cDNA provided by Dr R. Offringa (State University Leiden, The Netherlands).

Preparation of membrane fragments and ligand-blotting analysis

Frozen liver samples were triturated in liquid nitrogen in a mortar and solubilized (at 60 mg/ml) in PBS containing 0.05% (vol/vol) Tween 20. After homogenizing the suspension mechanically using a motor-driven Potter-Elvehjem homogeniser (10 strokes) at 0 °C, debris and nuclei were removed by centrifugation at 1000xg for 15 minutes. The supernatants, containing the membrane fragments, were stored at -20 °C until use. Samples (6 µl) were resolved by SDS-PAGE in a 5% (vol/vol) gel using an SDS-concentration of 0.1% (wt/vol). After electrophoresis, the proteins were transferred electophoretically to Protan nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) in a buffer of 25 mM Tris, 190 mM glycine, pH 8.6, 20% (vol/vol) methanol and 0.1% (wt/vol) SDS, at 1 mA/cm^2 overnight, using a Pharmacia-LKB semi-dryblott apparatus. The filters were blocked with 10 mM Tris, pH 8.0 with 150 mM NaCl, 1% (wt/vol) skim milk and 0.1% (vol/vol) Tween-20 for 1.5 hours, followed by incubation with ^{125}I-GST-RAP at a concentration of 2 nM in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% (wt/vol) skim milk and 0.1% (vol/vol) Tween-20 at room temperature for 3 hours. After incubation, the filters were washed extensively with 30 ml 10 mM Tris, pH 8.0, 150 mM NaCl and 0.1% (vol/vol) Tween-20 during 3 hours. Bound ligand was visualized by autoradiography.

Western blotting

For Western blotting, samples (7.5 µl) of membrane fragments were diluted 1 to 1 in Laemmli incubation buffer (50 mM Tris pH 6.8, 1% (wt/vol) SDS, 10% (vol/vol) glycerol and 8 M ureum) and separated on a 5-18% (wt/vol) Laemmli gel. After electrophoresis, the proteins were transferred to Protan nitrocellulose in a buffer of 192 mM glycine, 25 mM Tris (pH 8.3) and 10% (vol/vol) methanol at 300 mA/cm^2 overnight, using a wet-blot apparatus (Hoefer Scientific Instruments, San Francisco, CA). The filters were blocked with 1% (wt/vol) skim milk in buffer A consisting of 0.5 M NaCl, 20 mM Tris (pH 7.5) and 0.05% (vol/vol) Tween 20 for 0.5 hour at room temperature, followed by incubation with a mouse mannose receptor monoclonal antibody (Moab 15.2) at a concentration of 1 µg/ml in buffer A for 1.5 hours at room temperature. Next the blots were washed 3 times with buffer A and incubated for 1.5 hours at room temperature with rabbit anti-mouse RaM-PO (1:5000) (Nordic, Tilburg, The Netherlands) as a conjugate. Finally the blots were stained with the peroxidase substrate BM-blue (Boehringer-Mannheim, Mannheim, Germany).

RESULTS

Effect of estrogen treatment on plasma clearance of t-PA

To evaluate whether estrogen treatment influences the clearance of t-PA, we pursued two approaches. First, we determined the disappearance of exogenous recombinant human t-PA in ethinylestradiol (EE)-treated and control mice. As shown in Fig 1A for a bolus infusion of 15 μg of t-PA, clearance of t-PA was significantly faster in EE-treated mice than in control mice (0.46 ml/min versus 0.32 ml/min, respectively, $p< 0.01$). Secondly, we studied the clearance of bradykinin-released t-PA in EE-treated and control rats (Fig 1B). At baseline, before infusion of bradykinin, plasma t-PA antigen levels were 2.0 ng/ml and 2.5 ng/ml in EE-treated and control rats, respectively ($p< 0.05$). One minute after bradykinin injection, plasma t-PA antigen levels peaked in both experimental groups and were not significantly different in the EE-treated rats (8.7 ng/ml) versus the control rats (8.4 ng/ml), indicating that both groups started clearance with the same peak level of t-PA. t-PA antigen subsequently fell faster in the EE-treated group than in the control group (area under the curve (AUC) of 24.9 ng/ml.min and 31.9 ng/ml.min, respectively; $p<0.05$).

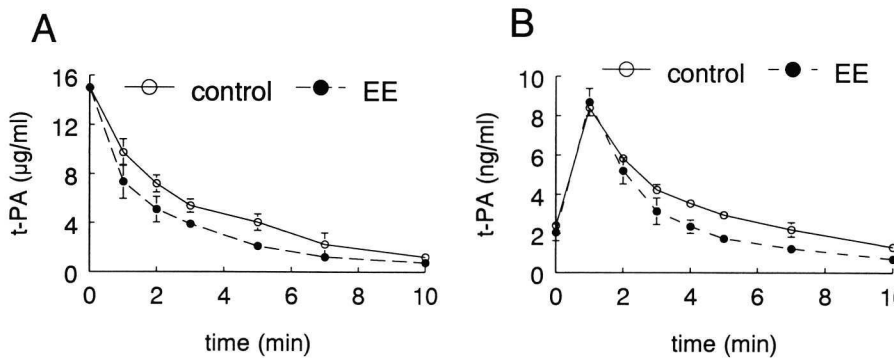

Figure 1 Effect of estrogen treatment on plasma clearance of t-PA. As described in Methods, mice were injected with 15 μg of recombinant human t-PA (A) or rats were injected with 50 μg/kg bodyweight bradykinin (B), after pretreatment of the animals with ethinylestradiol (EE) or vehicle (control). Blood samples were collected at the indicated times and t-PA antigen concentrations were determined. Data points are mean ± SD and represent one of three similar experiments done in five-fold (A) and one experiment done in triplicate (B).

Effect of RAP and mannan on the plasma clearance of t-PA

The clearance of t-PA from the circulation is mainly mediated via two independent receptor systems, the LRP and the mannose receptor. As a first approach to gain more insight into the receptor system responsible for the EE-induced increase in t-PA clearance, we blocked uptake of t-PA by each of the two receptor systems by specific antagonists.

Preinjection of GST-RAP (40 mg/kg), a dose previously shown to greatly block t-PA clearance via LRP in rats, (Biessen et al., 1997) reduced t-PA clearance in control (from 0.41 ml/min to 0.25 ml/min) and estradiol-treated mice (from 0.66 ml/min to 0.35 ml/min), but the difference in clearance of t-PA between the two experimental groups remained maintained (Fig 2). As a control, we also measured the clearance of radiolabelled α_2-macroglobulin, a specific ligand for LRP. Although the plasma disappearance of radiolabelled α_2-macroglobulin was strongly reduced by GST-RAP pretreatment, clearance of radiolabelled α_2-macroglobulin was not completely blocked, probably due to rapid clearance of RAP (results not shown).

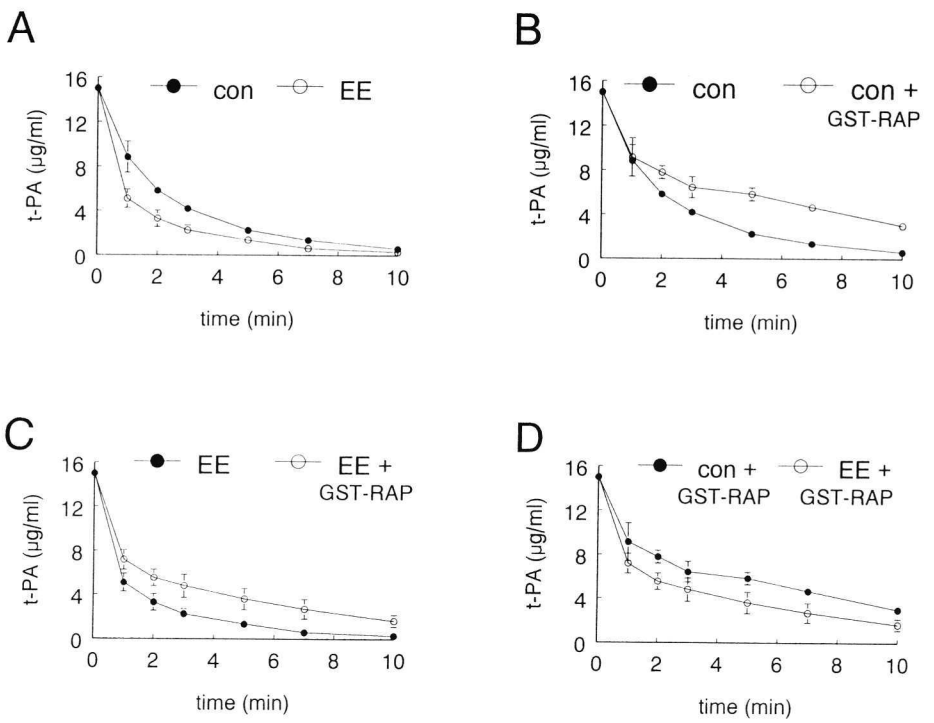

Figure 2 Effect of GST-RAP on the plasma clearance of t-PA in control and ethinylestradiol-treated mice. As described in Methods, ethinylestradiol (EE) or vehicle (con)-treated mice were injected with 15 µg of recombinant human t-PA, one minute after preadministration of 40 mg/kg bodyweight GST-RAP or PBS. Blood samples were collected at the indicated times and t-PA antigen concentrations were determined. Data points are mean ± SD of four or five mice in each treatment group. A: t-PA clearance in control and ethinylestradiol-treated mice. B: t-PA clearance in control mice, preinjected with GST-RAP or PBS. c: t-PA clearance in ethinylestradiol-treated mice, preinjected with GST-RAP or PBS. C: t-PA clearance in control and ethinylestradiol-treated mice, preinjected with GST-RAP.

Therefore, we also evaluated the functional effect of RAP expressed by adenoviral gene transduction. Five days after infection with Ad-RAP, EE-treated and control mice were unable to clear radiolabelled α_2- macroglobulin, whereas the control animals rapidly cleared this ligand for LRP (data not shown). In these Ad-RAP infected mice, in which clearance via LRP remains completely blocked, clearance of t-PA in the EE-treated animals was still increased as compared to control animals (0.23 ml/min versus 0.14 ml/min, $p< 0.01$) (Fig 3). These data are suggestive for upregulation of another receptor system, most likely the mannose receptor, to explain the increased t-PA clearance after EE-treatment.

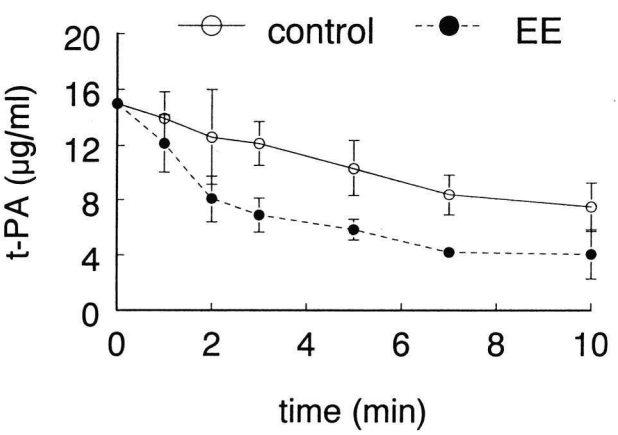

Figure 3 Effect of preadministration of AdCMV-RAP on plasma clearance of t-PA in control and ethinylestradiol-treated mice. As described in Methods, ethinylestradiol (EE) and vehicle (con)-treated mice were injected with AdCMV-RAP. Five days after virus administration, mice were injected with 15 µg recombinant human t-PA, and plasma t-PA concentrations were determined at the indicated times. Data points are mean ± SD of three mice in each treatment group.

To examine the contribution of the mannose receptor to the EE-induced t-PA clearance, we blocked this receptor system by preinjection of mice with mannan, a mannose receptor ligand. Pre-administration of mannan (5mg/kg), a dose previously shown to block clearance of ovalbumin, a mannose-terminated glycoprotein, reduced plasma clearance of t-PA from 0.32 ml/min to 0.22 ml/min in control mice, and from 0.46 ml/min to 0.24 ml/min in EE-treated mice (Figs 4A, B and C). Thus, in contrast to RAP-blockade of LRP, preadministration of mannan abolished the faster clearance of t-PA in EE-treated animals (Fig 4D). Together these data indicate that the estrogen-increased t-PA clearance is mediated via the mannan-inhibitable mannose receptor system, and not via the RAP-inhibitable LRP system.

Effect of ethinylestradiol on hepatic mannose receptor and LRP mRNA levels

Consistent with earlier observations in rats (Lund et al., 1989), we found hepatic LRP mRNA expression in mice almost exclusively in hepatocytes, whereas the mannose receptor mRNA was demonstrated mainly in the non-hepatocyte (Kupffer cell/endothelial cell) fraction (Fig 5). To further substantiate the EE-induced mannose receptor capacity, we performed

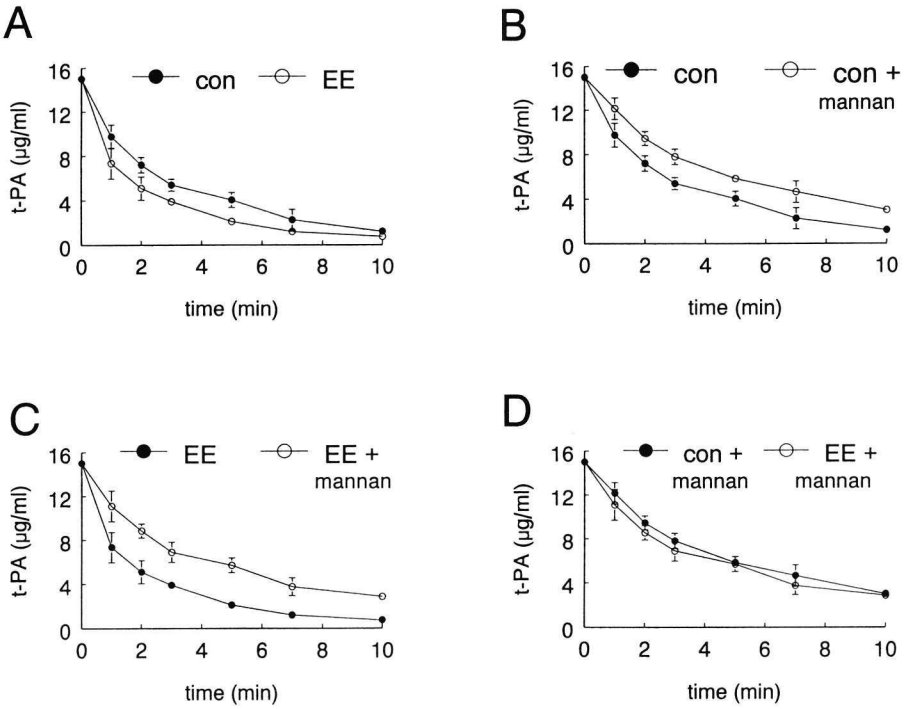

Figure 4 Effect of preadministration of mannan on the plasma clearance of t-PA in control and ethinylestradiol-treated mice. As described in Methods, ethinylestradiol (EE) or vehicle (con)-treated mice were injected with 15 µg of recombinant human t-PA, one to three minutes after preadministration of 5 mg/kg bodyweight mannan or PBS. Blood samples were collected at the indicated times and t-PA antigen concentrations were determined. Data points are mean ± SD of four or five mice in each treatment group.
A: t-PA clearance in control and ethinylestradiol-treated mice. B: t-PA clearance in control mice, preinjected with mannan or PBS. C: t-PA clearance in ethinylestradiol-treated mice, preinjected with mannan or PBS.
D: t-PA clearance in control and ethinylestradiol-treated mice, preinjected with mannan.

Northern-blotting studies to compare mannose receptor mRNA expression in livers from control and EE-treated mice. EE-treatment strongly increased liver mannose receptor levels (6.1 ± 1.1 fold, n=3), but not LRP mRNA levels (1.1 ± 0.1 fold induction, n=3)(Fig 6B). These effects of EE-treatment on the gene level were confirmed at the protein levels. As shown in Fig 7, Western blotting showed an increase in 180 kDa mannose receptor protein signal in a liver membrane preparation of EE-treated animals as compared with that of control animals. The amount of 600 kDa LRP protein, as determined by ligand blotting with ^{125}I RAP, showed no significant difference between the two groups. In these experiments, equal loading was controlled for by Ponceau coloring of the blots.

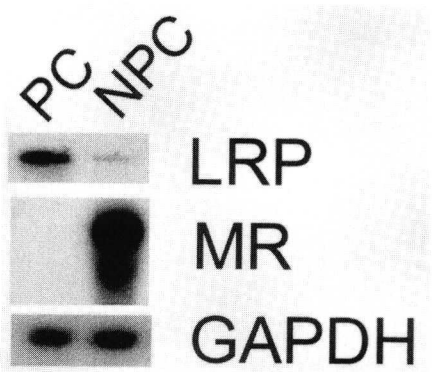

Figure 5 Expression of LRP and mannose receptor mRNA in parenchymal and non-parenchymal murine liver cells. As described in Methods, liver parenchymal (PC) and non-parenchymal cells (NPC) were isolated from control or ethinylestradiol-treated mice. Total RNA, was isolated and analyzed (15 µg/lane) by Northern blotting for LRP and mannose receptor (MR) mRNA levels, and as a reference for the amount of RNA in each lane, for GAPDH mRNA levels. The results shown are obtained with control mice. Comparable results were found with ethinylestradiol-treated mice (not shown).

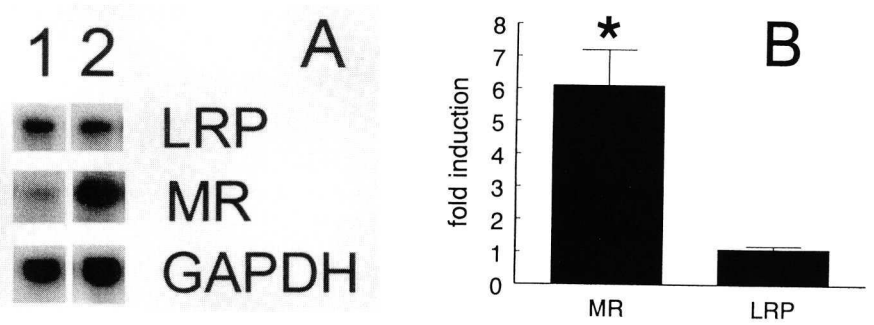

Figure 6 Effect of estradiol treatment on hepatic LRP and mannose receptor mRNA expression in mice. A: Total RNA was extracted from livers from ethinylestradiol (lane 2) or vehicle (lane 1)-treated mice, and analyzed by Northern blotting for LRP and mannose receptor (MR) mRNA levels. As a control for equal loading, the blots were probed with the cDNA for GAPDH mRNA. B: The signals for LRP and MR mRNA were quantified by phosphoimager analysis and adjusted for the corresponding GAPDH mRNA signals. The results shown are the amounts of hepatic LRP and MR mRNA in EE-treated mice relative to those found in vehicle-treated mice. Data are expressed as mean ± SD of three independent experiments consisting of at least four animals in each treatment group.

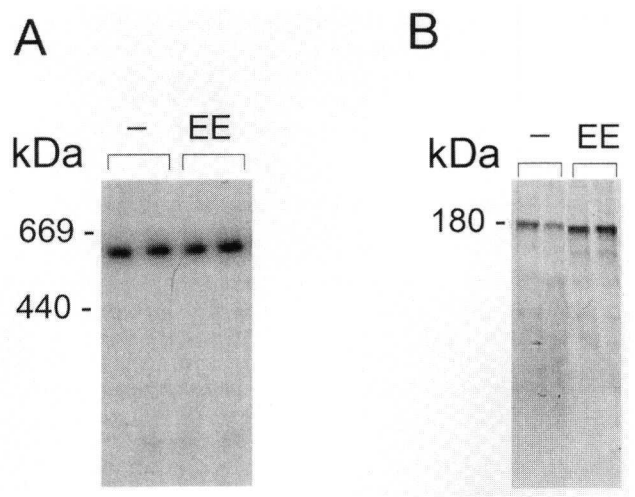

Figure 7 LRP and mannose receptor expression in livers from control and ethinylestradiol-treated mice. Hepatic membrane fragments from control and ethinylestradiol-treated mice were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, as described in the Methods section. LRP (panel A) was visualized by incubating the blot with ^{125}I GST-RAP. MR (panel B) was incubated with a monoclonal antibody against the MR and visualized as described in the Methods section.

DISCUSSION

The present study demonstrates that ethinylestradiol (EE) administration significantly increases the clearance of endogenous, bradykinin-released t-PA in rats and that of exogenously administered recombinant human t-PA in mice. This increased t-PA clearance after EE treatment is most likely the result of an increase in mannose receptor-mediated clearance. First, blocking of the LRP either by injection of GST-RAP or by overexpression of RAP by adenoviral gene transduction retarded but did not eliminate the difference in clearance between control and EE-treated mice, whereas in the presence of a mannose receptor antagonist, mannan, t-PA clearance in control and EE-treated mice became identical. Secondly, EE-treatment of mice induced a 6-fold increase in liver mannose receptor mRNA expression, while the amount of LRP mRNA in the EE-treated animals did not differ from that in control animals. These findings were confirmed at the protein level by Western blotting and ligand blotting, respectively.

An increased clearance of t-PA by estrogens as found in this study explains, at least in part, the inverse association between plasma t-PA levels and estrogens as observed in several studies. In a cross-sectional study, t-PA (and PAI-1) antigen levels were lower in premenopausal women than in age-matched men, with the sex difference disappearing after menopause (Gebara et al., 1995). t-PA and PAI-1 antigen levels were significant higher in postmenopausal than premenopausal women (Gebara et al, 1995; Shahar et al., 1996). Studies on the use of oral contraceptives (Gevers Leuven et al., 1987; Petersen et al., 1993), hormone replacement therapy (Gebara et al., 1995) by women or studies with male-to-female transsexuals (Kooistra et al., 1996) also show an inverse association between plasma t-PA

concentrations and estrogens. We can not exclude the possibility that other mechanisms may also contribute to the decreased t-PA antigen levels after estrogen treatment. For instance, estrogen administration is usually associated with lower plasma PAI-1 levels (Petersen et al., 1993; Quehenberger et al., 1993; Winkler et al., 1996). As a consequence, less t-PA/PAI-1 complex is formed, which may result in faster clearance of free t-PA (Chandler et al., 1997). The possibility that estrogens decrease plasma t-PA levels directly by decreasing endothelial t-PA production is not very likely, because *in vitro* studies with cultured human endothelial cells have shown that EE and 17-ß estradiol do not influence t-PA synthesis (Kooistra et al., 1990, 1996).

An effect of estrogens on the concentration of blood components via altered clearance is not unique for t-PA or the mannose receptor. Our finding that estrogens increase mannose receptor expression is able to explain the lower levels of other mannose receptor ligands in women as compared to men, like ß-glucuronidase and N-acetyl-ß glucosaminidase (Lombardo et al., 1981; Goi et al., 1993). Treatment of rats with EE leads to a marked increase in the number of LDL receptors, resulting in lower plasma LDL-cholesterol levels as a result of increased clearance (Hay et al., 1971; Kovane et al., 1979; Chao et al., 1979). To the same token, other compounds may change t-PA levels by modulating LRP expression. For example, LPS and interferon-γ have been demonstrated to downregulate, and dexamethasone to upregulate LRP expression (LaMarre et al., 1993; Kancha et al., 1996).

The effect of estrogens on mannose receptor expression may also have consequences for other cell types that express the mannose receptor, for instance mononuclear phagocytes. In these cell-types, the mannose receptor mediates the clearance of glycoproteins and microorganisms rich in mannose residues, which may be critical to the initial host defense reactions of macrophages (Schreiber et al., 1993). An increase in mannose receptor expression by estrogens may impact on this function. It is however uncertain whether the regulation of expression of the mannose receptor in these cells is similar to that in liver endothelial cells (Noorman and Rijken., 1997).

The molecular mechanism underlying the estrogen-induced mannose-receptor expression in mice was not the subject of the present study, but most probably involves estrogen receptor-mediated stimulation of mannose-receptor gene transcription. The recent description of a second estrogen receptor, named ERß (Kuiper et al., 1996), brings up many questions regarding the possibly distinct biological roles for the two estrogen receptor subtypes (α and ß), their tissue distribution and their ligand selectivities. In view of this, the regulation of the LDL receptor (mainly expressed in parenchymal cells) and the mannose receptor (in non-parenchymal cells) may involve different estrogen receptors, beit homodimeric forms of ERα or ERß or heterodimers (Cowley et al., 1997). In this context, it may also be noteworthy that the effects on mannose receptor expression, as described in this paper, were obtained using the synthetic estrogen, ethinylestradiol. In two recent studies it was found that oral

administration of conjugated estrogens reduced PAI-1 levels by about 50%, whereas, by contrast, transdermal 17ß-estradiol led to no significant changes in PAI-1 (Kroon et al., 1994, Koh et al., 1997). One explanation for this difference would be that oral administration results in the presence of estrogen in sufficiently high concentrations in the portal circulation, after absorption from the gut, to exert hepatic effects. Alternatively, synthetic ethinylestradiol and naturally occuring 17ß-estradiol may show different relative affinities for ERα versus ERß (Kuiper et al., 1997).

In conclusion, the increased t-PA clearance after estrogen treatment via increased mannose receptor expression as shown in this study provides an explanation for the lowered t-PA levels associated with high estrogen status. The data presented in this study demonstrate for the first time that estrogens, besides their well-known effects on plasma lipid levels via changes in clearance receptors, are also able to modulate the haemostatic system via changes in clearance rate. Further research, including identification of the estrogen receptor(s) involved, is required to precisely analyze the estrogen-regulated mannose receptor gene expression at the molecular level.

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CHAPTER 6

EFFECT OF STEROID HORMONES AND RETINOIDS ON THE FORMATION OF CAPILLARY-LIKE TUBULAR STRUCTURES OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS IN FIBRIN MATRICES IS RELATED TO UROKINASE EXPRESSION

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ABSTRACT

Angiogenesis, the formation of new capillary blood vessels, is a feature of a variety of pathological processes. To study the effects of a specific group of hormones (all ligands of the steroid/retinoid/thyroid hormone receptor superfamily) on the angiogenic process in man, we have used a model system in which human microvascular endothelial cells from foreskin (hMVEC) are cultured on top of a human fibrin matrix in the presence of basic fibroblast growth factor and tumor necrosis factor α. This model mimics the *in vivo* situation where fibrin appears to be a common component of the matrix present at sites of chronic inflammation and tumor stroma. Our results demonstrate that testosterone and dexamethasone are strong inhibitors and all-*trans* retinoic acid (*at*-RA) and 9-*cis* retinoic acid (9-*cis* RA) are potent stimulators of the formation of capillary-like tubular structures. These effects are mediated by their respective nuclear hormone receptors as demonstrated by the use of specific synthetic receptor agonists and antagonists. 17ß-Estradiol, progesterone and 1,25- dihydroxyvitamin D3 did not affect or only weakly affected *in vitro* angiogenesis, which may be related to the lack of significant nuclear receptor expression. Although hMVEC express both thyroid hormone receptors α and ß, no effect of thyroid hormone on tube formation was found. The effects of testosterone, dexamethasone, *at*-RA and 9-*cis* RA on tube formation were accompanied by parallel changes in urokinase-type plasminogen activator (u-PA) expression, at both mRNA and antigen levels. Exogenous suppletion of the medium with single chain u-PA enhances tube formation in our *in vitro* model, whereas quenching of u-PA activity (but not of tissue-type plasminogen activator activity) or of u-PA binding to u-PA receptor by specific antibodies suppressed basal and retinoid-stimulated tube formation. Moreover, addition of scu-PA to testosterone- or dexamethasone-treated hMVEC restored the suppressed angiogenic activity for a substantial part. Our results demonstrate that steroid hormones (testosterone and dexamethasone) and retinoids have strong, but opposite effects on tube formation in a human *in vitro* model reflecting pathological angiogenesis in the presence of fibrin and inflammatory mediators. These effects can be explained by hormone-receptor-mediated changes in u-PA expression.

INTRODUCTION

Angiogenesis is the formation of new capillary blood vessels by a process of sprouting from existing microvascular vessels. It has a role during development and in the normal physiology of reproduction, formation of collaterals, and wound healing, but is also important under pathological conditions, where it contributes to the pathogenesis of a number of diseases such as diabetic retinopathy, tumor growth and rheumatoid arthritis (1-5). In each system, the formation of new capillaries involves a series of discrete, but overlapping events, including (i) localized degradation of the endothelial cell basal lamina; (ii) endothelial cell migration and

extracellular matrix invasion; (iii) endothelial cell proliferation; (iv) formation of capillary lumina and reconstitution of the basal lamina (6,7). Given the (patho)physiological importance of angiogenesis, it is of clinical relevance to identify factors that either stimulate or inhibit those processes, and to elucidate their mode of action.

Several reports have pointed to an effect of steroid hormones and retinoids (retinoic acid derivatives) on angiogenic activity. However, results have been conflicting, and both stimulatory and inhibitory activities of these compounds have been found (8-17). It is not clear at present, whether the variation in response to hormones relates to differences in assay systems, species, the parameter measured, or hormone concentration and metabolism. Many studies have been performed in non-human angiogenesis models and focused on individual components of the angiogenic process (for instance, proliferation or migration), rather than on the complete response of capillary tube formation. To gain more insight into the effect of hormones on angiogenesis in humans, we have developed an *in vitro* model, in which human foreskin microvascular endothelial cells (hMVEC) can be induced to invade a three-dimensional fibrin matrix thereby forming capillary-like tubular structures, which can be quantified by computer-assisted video analysis (18). In this *in vitro* model, both a growth factor (basic fibroblast growth factor (bFGF) or vascular endothelial cell growth factor) and a factor to induce urokinase-type plasminogen activator (u-PA), for example tumor necrosis factor α (TNFα), are necessary to induce capillary-like tubular structures. This model mimics the *in vivo* situation where fibrin appears to be a common component of the matrix present at sites of chronic inflammation and tumor stroma (19). Electron microscopy of cross sections showed that the capillary-like tubular structures have a lumen and are very much like capillary structures *in vivo* (18). Tube-formation in this model was shown to be dependent on u-PA activity and plasmin activity, which are thought to play a role in the regulation of the first steps of angiogenesis.

We have used this *in vitro* model to examine the angiogenesis-modulating activity of a group of hormones acting via binding to a specific class of nuclear receptors, the steroid/retinoid/thyroid hormone receptor superfamily (20). Our results demonstrate both blocking (testosterone and dexamethasone) and stimulating (all-*trans* and 9-*cis* retinoic acid) activities of the hormones tested. In all cases, hormone-induced changes in tube formation are paralleled and can be mimicked by corresponding changes in u-PA levels. Our findings provide evidence for an important role for hormones and their respective nuclear receptors in the formation of tube-like structures by influencing the expression of u-PA.

MATERIALS AND METHODS

Materials

17ß-estradiol, 2-methoxyestradiol, progesterone, testosterone, dexamethasone, 3,3,5-triiodo-l-thyronine (T3), all-*trans* retinoic acid (*at*-RA), dimethyl sulfoxide (DMSO) and charcoal-stripped, delipidated bovine calf serum were purchased from Sigma (St Louis, MO). R1881 (methyltrienolone) was obtained from NEN (Boston, MA). 1,25-Dihydroxyvitamin D3 (Ro 21-5535) was kindly provided by Dr M.R. Uskoković (Hoffmann-LaRoche, Nutley, NJ) and 9-*cis* retinoic acid (9-*cis* RA) and the retinoic acid receptor α (RARα) antagonist, Ro 41-5253, were kindly provided by Drs M. Klaus and C. Apfel (Hoffmann-LaRoche, Basel, Switzerland). Hydroxyflutamide was a gift from Dr T. Lavecchia (Schering-Plough, Kenilworth, NJ). 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphtyl)-ethenyl] benzoic acid (3-methyl-TTNEB) and (E)-4-[2-(5,5,8,8,-tetramethyl-5,6,7,8-tetrahydro-2-naphtalenyl-1-propenyl] benzoic acid (TTNPB) were kindly provided by Dr S. Karathanasis, American Cyanamid Company (Pearl River, NY) and by Dr M. Issandou, Glaxo Wellcome (Les Ulis, France), respectively. Stock solutions of hormones (10 mmol/L) were prepared in DMSO and stored at -20 °C. Stock solutions were diluted with incubation medium to the final test concentations immediately before the start of an experiment. All experiments involving retinoids, R1881 and hydroxyflutamide were performed in subdued light, and the tubes containing these test compounds were covered with aluminium foil. bFGF was obtained from Intergen (Purchase, NY), thrombin from Leo Pharmaceutical Products (Weesp, The Netherlands) and human fibrinogen from Chromogenix AB (Mölndal, Sweden). Factor XIII was kindly provided by Dr H. Keuper (Behringwerke, Marburg, Germany). Human recombinant TNFα was a gift from Dr J. Travernier (Biogent, Ghent, Belgium), and contained $2.45x10^7$ U/mg protein and <40 ng lipopolysaccharide per µg protein. Single chain u-PA was generously provided by Dr A. Molinari (Farmitalia, Milan, Italy). Rabbit polyclonal anti-u-PA antibodies and rabbit polyclonal anti-t-PA antibodies were prepared in our laboratory. The u-PA receptor (u-PAR) blocking monoclonal antibody H-2 was a gift from Dr U. Weidle (Boehringer-Mannheim, Penzberg, Germany). Technovit 8100 was obtained from Heraeus Kulzer (Wehrheim, Germany). ELISA kits for determination of t-PA antigen (Thrombonostika t-PA) and PAI antigen (Imulyse) were obtained from Organon Teknika (Boxtel, The Netherlands) and Biopool (Umeå, Sweden), respectively. Deoxycytidine 5[α-^{32}P] triphosphate was from Amersham (Buckinghamshire, UK). Oligonucleotides used for RT-PCR were purchased from Isogen Bioscience (Maarssen, The Netherlands). Other materials used have been specified in the methods described or in the related references.

Cell culture

Human foreskin microvascular endothelial cells (hMVEC) were isolated, cultured and characterized as previously described (21). The cells were cultured in gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mmol/L HEPES (pH, 7.3), 10% (vol/vol) human serum, 10% (vol/vol) heat-inactivated newborn calf serum (NBCS), 150 µg/mL crude endothelial growth factor (22), 5 IU/mL heparin, 2mmol/L L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. Subcultures were obtained by trypsin/EDTA treatment of confluent monolayers at a split ratio of 1:3.

In vitro angiogenesis model

Human fibrin matrices were prepared by addition of 0.1 U/mL thrombin to a mixture of 2.5 U factor XIII, 2 mg fibrinogen, 2 mg Na-citrate, 0.8 mg NaCl and 3 µg plasminogen per mL DMEM medium without indicator. 300 µL of this mixture was added to 1 cm^2 wells. After clotting at room temperature, the fibrin matrices were soaked with 0.5 mL DMEM supplemented with 10% (vol/vol) heat-inactivated human serum, 10% (vol/vol) heat-inactivated NBCS and penicillin/streptomycin. Endothelial cells were seeded at high density to obtain confluent monolayers, and, unless stated otherwise, cultured in DMEM without indicator supplemented with 10% (vol/vol) heat-inactivated human serum, 10% (vol/vol) heat-inactivated NBCS, 2mmol/L L-glutamine, penicillin/streptomycin, 20 ng/mL bFGF and 20 ng/mL TNFα (referred to as incubation medium). Incubations were for 8-12 days, and test compounds were added together with incubation medium where appropiate. The conditioned medium was collected and replaced every 2 to 3 days. Invading cells and the formation of capillary-like tubular structures ("tube formation") of endothelial cells in the three-dimensional fibrin matrix were analyzed by phase contrast microscopy and the total number, the total area and the total length of capillary-like tubular structures of six randomly chosen microscopic fields/well (11.6 mm^2/field) were measured using an Olympus microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

Because all three measured parameters correlated well with each other (r>0.96), only data for total length of capillary-like structures are shown. For determination of t-PA secretion, cells were cultured on gelatin-coated 1 cm^2 wells in parallel to cells grown on fibrin matrices. In experiments with nuclear receptor antagonists, the antagonist was added to the medium one hour before the hormone.

Histochemistry

Matrices were fixed in 2% (vol/vol) p-formaldehyde in phosphate buffered saline (PBS) (0.15 mol/L NaCl/10 mmol/L Na_2HPO_4/1.5 mmol/L KH_2PO_4, pH 7.4), embedded in Technovit 8100, sectioned at 3 µm and stained with hematoxylin and floxin.

ELISAs

u-PA antigen was determined by a previously described u-PA ELISA which recognizes single-chain u-PA, two-chain u-PA and u-PA/PAI-1 complex with the same efficiency (18). t-PA and PAI-1 antigen determinations were performed by commercially available immunoassay kits (Thrombonostika t-PA and IMULYSE™).

Northern blot analysis

Total RNA from hMVEC (30 cm^2) was isolated by the isothiocyanate/phenol/acid extraction method of Chomzynski et al. (23). The RNA was dissolved in H_2O, and the RNA concentration was determined spectrophotometrically. Equal amounts (7.5 µg) of RNA were separated on a formaldehyde/agarose gel and were subsequently capillary transferred to a Hybond N membrane according to the instructions of the manufacturer (Amersham). Hybridization was performed in 7% (wt/vol) sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, 0.5 mol/L Na_2HPO_4/NaH_2PO_4 buffer, (pH, 7.2) overnight at 63 °C with 25 ng of probe labeled with the random primer method (Megaprime kit, Amersham). The membranes were subsequently washed three to four times during 20 minutes with 2xSSC/1-%(wt/vol) SDS (1xSSC: 0.15 mol/L NaCl, 0.015 mol/L Na_3citrate) when using the u-PA and u-PAR probes, and twice with 2xSSC and twice with 1xSSC when using the actin probe. The filters were exposed to an Amersham Hyperfilm-MP film with an intensifying screen at -80 °C.

cDNA probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1023 bp fragment of the human u-PA cDNA (a gift from Dr W-D. Schleuning, Schering AG, Berlin, Germany)(24), a 585 bp BamHI fragment of the human u-PAR cDNA (25) (a kind gift from Dr F. Blasi, Milano, Italy) and a 1200 bp *Pst* I fragment of hamster actin cDNA (26).

Oligonucleotide primers

The following primer sequences were used in the reverse transcriptase-PCR (RT-PCR) to detect receptor mRNA: for the estrogen receptor α mRNA: sense 5'-TGATGGGGAGGGCAGGGGTGAAGTG-3' (aa 272-279) and antisense 5'-TAGGCGGTGGGCGTCCAGCATCTCC-3'(aa 541-549), as described by Perrot-Applanat et al.(27). For the estrogen receptor ß mRNA: sense 5'-TTGTGCGGAGACAGAGAAGTGC-3' (aa 175-182) and antisense 5'-GGAATTGAGCAGGATCATGGCC-3'(aa 349-355) (28). For the progesterone receptor mRNA: sense 5'-GTGGGCGTTCCAAATGAAAGCCAAG-3' (aa 660-667) and antisense 5'-QAATTCAACACTCAGTGCCCGGGACT-3' (aa 897-905) (27). For the thyroid receptor α mRNA: sense 5'-AGTGGGATCTGATCCACATTGC-3' (aa 164-171) and antisense 5'-GATCTTGTCCACACACAGCAGG-3' (aa 332-338)(29). For the thyroid receptor ß: sense 5'-GGGAGCTCATCAAAACTGTCAC-3' (aa 214-221) and antisense 5'-GGCTACTTCAGTGTCATCCAGG-3'(aa 359-366) (30). For the vitamin D3 receptor: sense 5'-AGACACACTCCCAGCTTCTCTG-3' (aa 171-180) and antisense 5'-ACGTCTGCAGTGTGTTGGACAG-3' (aa 359-366) (31).

RT-PCR

RT-PCR was performed under standard conditions following the specifications recommended by the supplier. In short, cDNAs were synthesized in one reaction mixture containing 1 µg total RNA, 0.45 µg oligo dT primer and RT-II-superscript (Life Technologies, Paisley, UK); then the cDNAs were heated for 8 minutes at 95 °C. Subsequently, the cDNAs were treated with RNAse H (25 U/mL) for 25 minutes at 37°C. Next, the cDNAs (1 µL of a 10x diluted cDNA reaction mixture) were amplified in the presence of 5% (vol/vol) DMSO and 5% W-1 (vol/vol)(Life Technologies) with the corresponding primers. The amplifications were performed for 30 cycles for

all receptors. The denaturation was performed for 60 seconds at 94 °C. Primer extension was performed for 60 seconds at 60 °C for 4 cycles, then at 58 °C for 4 cycles, next at 56 °C for 4 cycles and finally at 55°C for 18 cycles for the progesterone receptor and the thyroid receptors α and ß. For the estrogen receptors α and ß and for the vitamin D receptor, primer extension was performed for 60 seconds at 60 °C for 10 cycles and then at 58 °C for 20 cycles. The DNA-synthesizing step was performed at 72°C for 1 minute. For the estrogen receptors α and ß PCR was also performed for 55 cycles, with a cycling profile of 1 minute at 94 °C, 1 minute at 62 °C and 1 minute at 72 °C. After 20 cycles with a primer concentration of 28 nM additional primers were added to reach a final concentration of 380 nM. Aliquots of the PCR reaction mixture were separated on an agarose gel, stained with ethidium bromide, and visualised with a UV transilluminator.

RESULTS

Effect of steroid hormones, retinoids, thyroid hormone and 1,25-dihydroxyvitamin D3 on tube formation

To determine the effect of the various hormones on tube formation, hMVEC were seeded on three-dimensional fibrin matrices and maintained in standard incubation medium containing 10% (vol/vol) human serum, 10% (vol/vol) NBCS, 20 ng/ mL TNFα and 20 ng/mL bFGF, and varying concentrations of the appropiate hormone. Tube formation required the presence of both bFGF and TNFα, and none of the hormones tested showed any angiogenic activity by itself or in combination with either bFGF or TNFα (data not shown). Tube formation usually became detectable after 3 to 5 days of cell culture, and the onset of this process was not or hardly affected by the presence of the hormones. Effects of hormones on tube formation were most apparent after 8 to 12 days of culture and are illustrated in Figure 1 (A-E) for the most potent compounds, viz. *at*-RA, testosterone and dexamethasone. Very similar results were obtained when the human serum/ NBCS was replaced by 20% (vol/vol) delipidated calf serum (data not shown). Histological analysis of cross-sections perpendicular to the surface of the matrix shows that the tube-like structures are located in the fibrin matrix underneath the endothelial cell monolayer (Fig 1F). At a hormone concentration of 1 µmol/L, tube formation was strongly suppressed by testosterone and dexamethasone to 32 ± 9% and 30 ± 17% of control values, respectively (Fig 2). The synthetic androgen receptor agonist R1881 also effectively inhibited tube formation (see Fig 6A). 1 µmol/L 17ß-estradiol and its metabolite, 2-methoxyestradiol, only weakly inhibited tube formation to 82 ± 11% and 74 ± 19%, respectively, whereas progesterone (1µmol/L) had no significant effect (85 ± 17%) (Fig 2). *at*-RA (a ligand for the retinoic acid receptor, RAR) and 9-*cis* RA (a ligand for both RAR and the retinoid X receptor, RXR) both stimulated tube formation, with 9-*cis* RA being an even more potent stimulator (377 ± 80%) than *at*-RA (278 ± 40%) (Fig 2B). Comparable results were obtained with the RAR- specific synthetic ligand TTNPB and the RXR-specific ligand TTNEB (data not shown), indicating that both RARs and RXRs are able to mediate the effect of retinoids on tube formation. Neither thyroid hormone (T3) nor 1,25-dihydroxyvitamin D3 significantly affected tube formation at a concentration of 1 µmol/L (Fig 2B), but at a concentration of 10 µmol/L 1,25-dihydroxyvitamin D3 increased tube formation to 161 ± 35% (data not shown). Effective hormones influenced tube formation in a

concentration-dependent manner with ED_{50} values of 20 nmol/L for testosterone, 8 nmol/L for dexamethasone, 60 nmol/l for *at*-RA and 70 nmol/L for 9-*cis* RA, and maximal effects were reached at hormone concentrations of 0.1 to 1.0 μmol/L.

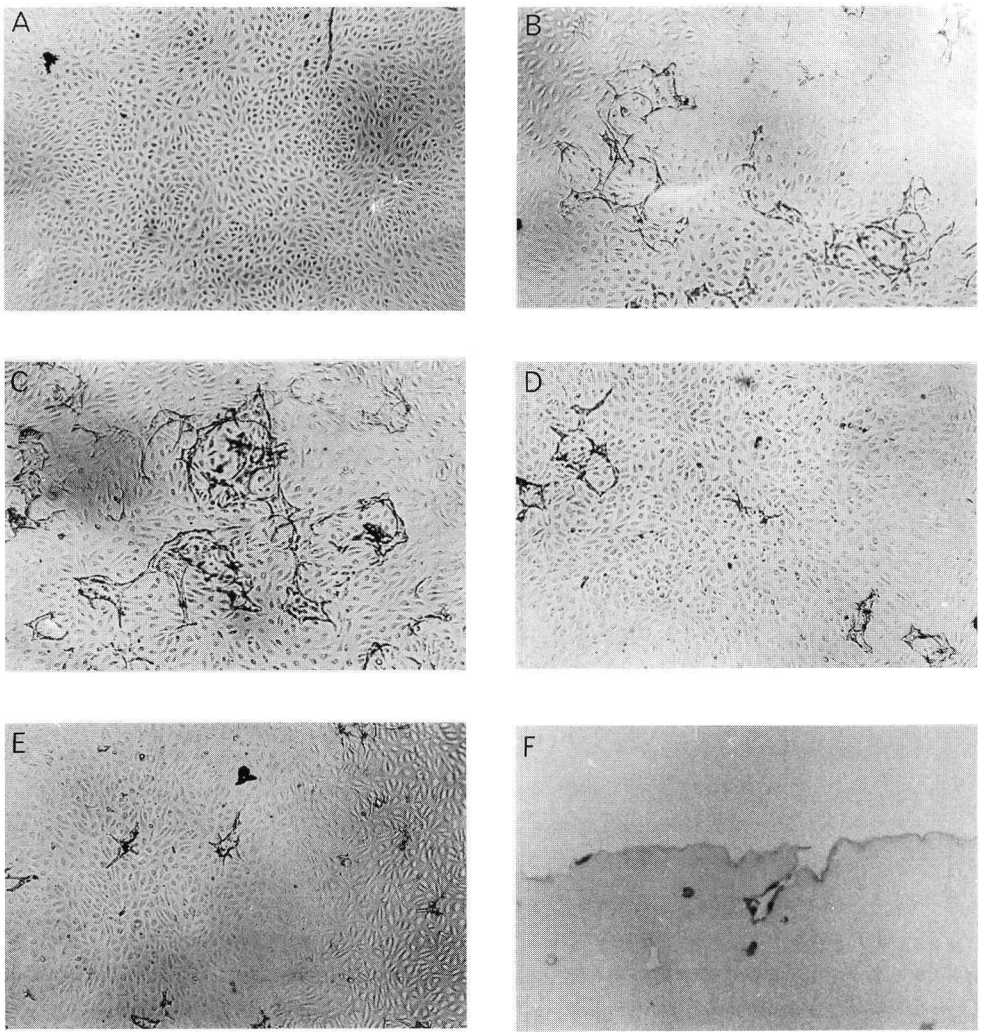

Figure 1. Effect of all-*trans* retinoic acid, testosterone and dexamethasone on *in vitro* angiogenesis. Human microvascular endothelial cells were cultured on the surface of a three-dimensional fibrin matrix in incubation medium containing 20 ng/mL TNFα and 20 ng/mL bFGF, supplemented with hormone or vehicle. After 9 days of culture, nonphase contrast photographs were taken with the plane of focus beneath the endothelial surface monolayer. A: incubation medium from which bFGF and TNFα had been omitted; B: incubation medium + vehicle [0.01% (vol/vol) DMSO]; C: incubation medium + all- *trans* retinoic aicd (1 μmol/L); D: incubation medium + testosterone (1 μmol/L); E: incubation medium + dexamethasone (1 μmol/L); F: incubation medium + vehicle; cross-section through a fibrin matrix perpendicular to the surface of the matrix, stained with hematoxylin and phloxin.

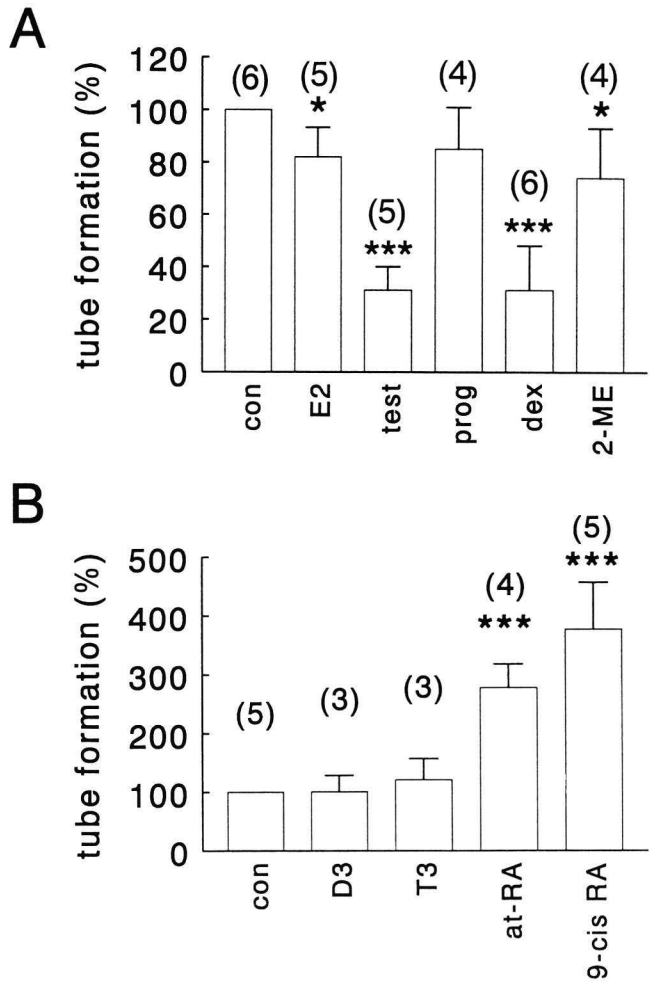

Figure 2. Effect of steroid hormones, retinoids, thyroid hormone and 1,25-dihydroxyvitamin D3 on tube formation. Human microvascular endothelial cells were cultured on the surface of a three-dimensional fibrin matrix in incubation medium containing 20 ng/mL bFGF and 20 ng/mL TNFα. Panel A shows the effect of vehicle, 0.01% (vol/vol) DMSO (con) or 1μmol/L 17ß-estradiol (E2), testosterone (test), progesterone (prog), dexamethasone (dex) or 2-methoxyestradiol (2-ME) and panel B the effect of 1 μmol/L of 1,25-dihydroxyvitamin D3 (D3), thyroid hormone (T3), all-*trans* retinoic acid (at-RA) or 9-*cis* retinoic acid (9-cis RA) on tube formation. The data represent the average of 3-6 experiments with the number of experiments indicated in parentheses for each condition, with each experiment performed in duplicate, and are expressed as percentage of control values. Total tube-length/cm^2 under control conditions ranged from 32 to 329 mm/cm^2 (average=130 mm/cm^2)) between the different experiments.
* $p<0.05$, *** $p< 0.0001$

Role of u-PA in hormone-modulated tube formation

Because we had previously demonstrated that tube formation in our *in vitro* angiogenesis model depends on u-PA activity (18), we evaluated the effect of the various hormones on the accumulation of u-PA in the medium (Fig 3). Under standard incubation conditions, a continuous increase in u-PA accumulation rate was observed from day 2 onwards over a 9 day period (Fig 3A and B). At a concentration of 1 μmol/L, testosterone and dexamethasone lowered u-PA accumulation to 66 ± 4% and 52 ± 6% of control values respectively after 9 days, *at*-RA and 9-*cis* RA enhanced the u-PA accumulation to 236 ± 56% and 284 ± 18%, respectively (Fig 3A and B).

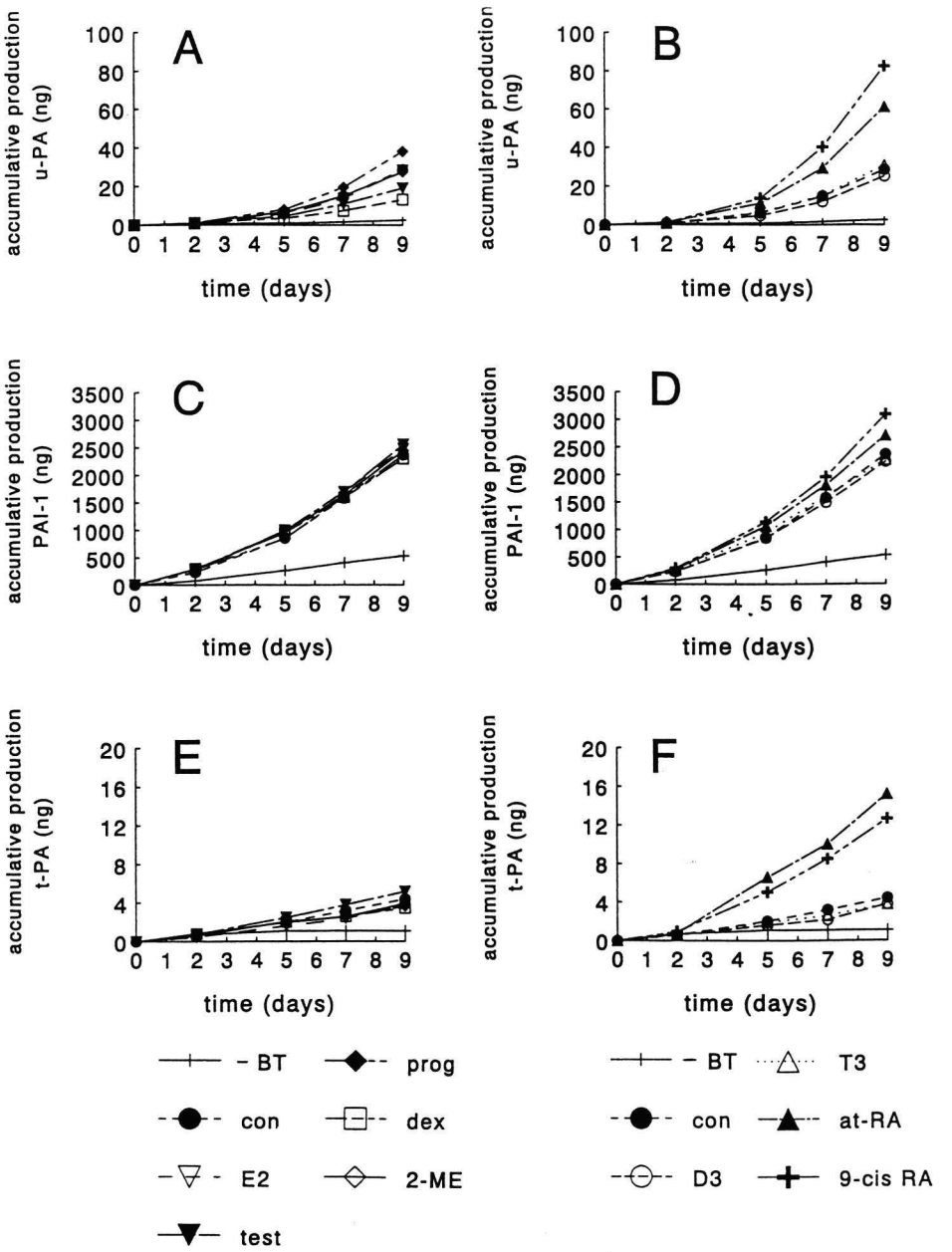

Figure 3. Accumulation of u-PA, PAI-1 and t-PA in hormone-treated human microvascular endothelial cells conditioned medium. Human microvascular endothelial cells were cultured on three-dimensional fibrin matrices (for determination of u-PA and PAI-1 production) or gelatin-coated dishes (for determination of t-PA production) in incubation medium containing 20 ng/mL bFGF and 20 ng/mL TNFα in the presence of vehicle (con) or the indicated hormones (1 μmol/L). Panels A and B show the accumulative production in the medium of u-PA

(ng/well), panels C and D the accumulative production of PAI-1 (ng/well) and panels E and F the accumulative production of t-PA (ng/well). u-PA, PAI-1 and t-PA antigen were determined in the conditioned medium by ELISA as described in Materials and Methods. The data shown are from one representative experiment (out of 5 performed). Under control conditions, the cumulative levels of u-PA, PAI-1 and t-PA after 9 days of culture varied between 20-49 ng/ well, 2300-2440 ng/well and 3.4-5.4 ng/well, respectively in the different experiments. For comparison, u-PA, PAI-1 and t-PA antigen levels were also determined when cells were cultured in incubation medium from which bFGF and TNFα had been omitted (-BT). E2: 17ß-estradiol; test: testosterone; prog: progesterone; dex: dexamethasone; 2-ME:2-methoxyestradiol; D3: 1,25-dihydroxyvitamin D3; T3: thyroid hormone; at-RA: all-*trans* retinoid acid; 9-cis RA; 9-*cis* retinoic acid.

The other hormones tested, 17ß-estradiol, 2-methoxyestradiol, progesterone, T3 and 1,25-dihydroxyvitamin D3, did not change u-PA production significantly in our *in vitro* model. The effect of the various hormones on u-PA accumulation thus highly parallels the effect on tube formation. Similarly as found for u-PA, PAI-1 accumulation rate increased in time. Of all the hormones tested, only *9-cis* RA and *at*-RA slightly increased PAI-1 levels (Figs 3C and D). Because t-PA binds to fibrin, we performed parallel experiments with hMVEC grown on gelatin-coated dishes to evaluate the effect of the various experimental conditions on t-PA accumulation. t-PA production proceeded at a constant rate. Both *at*-RA and 9-*cis* RA stimulated t-PA production to 374 ± 91% and 278 ± 25% of control values, respectively, whereas the other compounds did not affect t-PA production (Figs 3E and F). Omitting bFGF and TNFα from the standard incubation medium resulted in a very low, constant production rate of u-PA, PAI-1 and t-PA. In all, these findings are consistent with a role of u-PA (but not of t-PA) in mediating the hormonal effect on tube formation.

To further substantiate that hormonal modulation of tube formation in our *in vitro* model is related to changes in u-PA levels, we performed experiments in which the amount of u-PA available for angiogenesis was altered by the addition of specific antibodies or exogenous single chain u-PA (scu-PA). Figure 4A shows that the addition of antibodies which neutralize u-PA activity, completely inhibited basal and *at*-RA-stimulated tube formation, whereas antibodies which neutralize t-PA activity were without effect. Similarly, antibodies directed against u-PAR strongly inhibited basal (by 75 ± 26%) and *at*-RA-stimulated tube formation (by 68 ±19%)(Fig 4B). The structures which were formed in the presence of u-PAR antibody were very small and resembled single invading cells rather than capillary-like tubes. The addition of exogenous scu-PA to control cultures (mimicking retinoid-stimulated u-PA production) dose-dependently increased tube formation, and exogenous scu-PA could also restore for a great part the testosterone- and dexamethasone-inhibited tube formation (Fig 4 C). Together these findings indicate that differences in u-PA levels explain the observed hormone effects on tube formation.

u-PA and u-PAR mRNA expression

Northern-blotting studies were conducted to examine whether the effect of hormones on u-PA protein levels were reflected at the mRNA level. After a 96-hour exposure of hMVEC to the various hormones, testosterone and dexamethasone were found to decrease u-PA mRNA levels

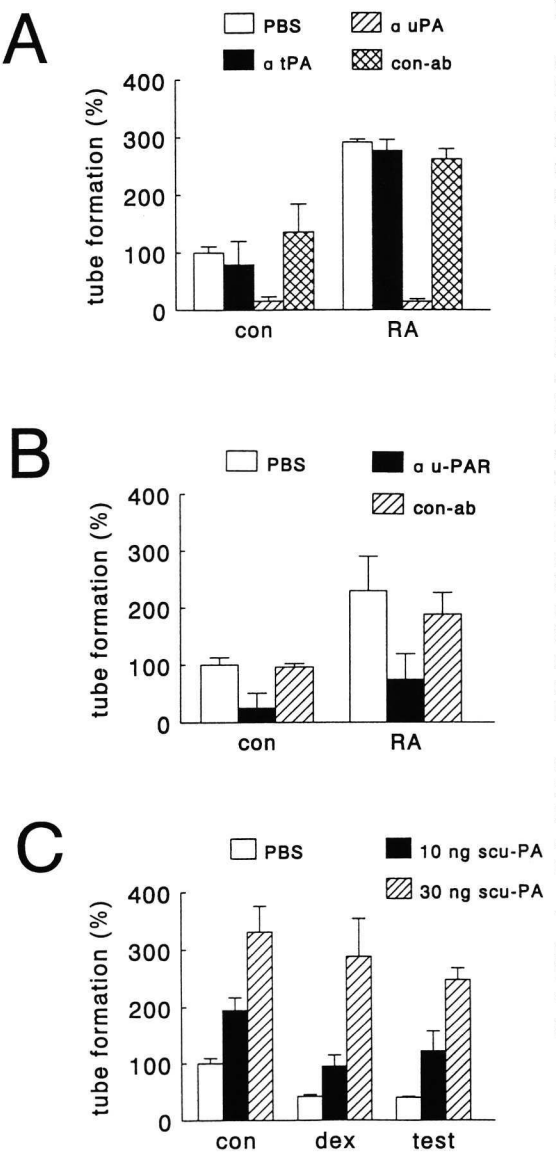

Figure 4. Effects of antibodies against u-PA, t-PA and u-PAR and of addition of single chain u-PA on hormone-modulated tube formation. Human microvascular endothelial cells were cultured on three-dimensional fibrin matrices in incubation medium containing 20 ng/mL bFGF and 20 ng/mL TNFα, and the appropiate hormone. Activity blocking antibodies to u-PA, t-PA and u-PAR were added at the start of an experiment, whereas single chain u-PA was added from day 2 onward. After 8-11 days the total length of capillary-like tubular structures was determined as described in Materials and Methods.

A: Effect of solvent (PBS), antibodies to u-PA (α uPA; 150 μg/mL) or t-PA (α tPA; 150 μg/mL) and rabbit IgG isolated from pooled normal serum (con-ab; 150 μg/ml) on tube formation of human microvascular endothelial cells in incubation medium supplemented with vehicle (con; 0.01% (vol/vol) DMSO) or all-*trans* retinoic acid (RA; 1 μmol/L).

B: Effect of solvent (PBS), antibody to u-PAR (α uPAR; 25 μg/mL) and antibody to FITC (con-ab; 25 μg/mL) on tube formation of human microvascular endothelial cells in incubation medium supplemented with vehicle (con; 0.01% DMSO) or all-*trans* retinoic acid (RA; 1 μmol/L).

C: Effect of addition of solvent (PBS) and scu-PA (10 or 30 ng scu-PA) on tube formation of human microvascular endothelial cells in incubation medium supplemented with vehicle (con; 0.01% DMSO), testosterone (test; 1 μmol/L) or dexamethasone (dex; 1 μmol/L).

The data represent the average of 2 experiments performed in duplicate wells (with ranges given by error bars) and are expressed as percentages of control values (tube-length/cm^2 in the presence of bFGF and TNFα).

by approximately 2- fold, whereas *at*-RA and 9-*cis* RA increased u-PA mRNA levels 2- and 3-fold, respectively (Fig 5). The other hormones did not significantly influence u-PA mRNA levels. Hybridization with the cDNA for u-PAR revealed a very similar induction pattern, with the exception of dexamethasone, which did not affect u-PAR mRNA levels. Northern-blotting analysis of u-PA and u-PAR mRNA expression after 8 hours of incubation very much resembled that of the 96-hour hormone exposure, except for testosterone which did not cause any early

change in u-PA and u-PAR mRNA expression (data not shown). Omitting bFGF and TNFα from the incubation medium resulted in a 2- to 3- fold decrease in u-PA and u-PAR mRNA levels.

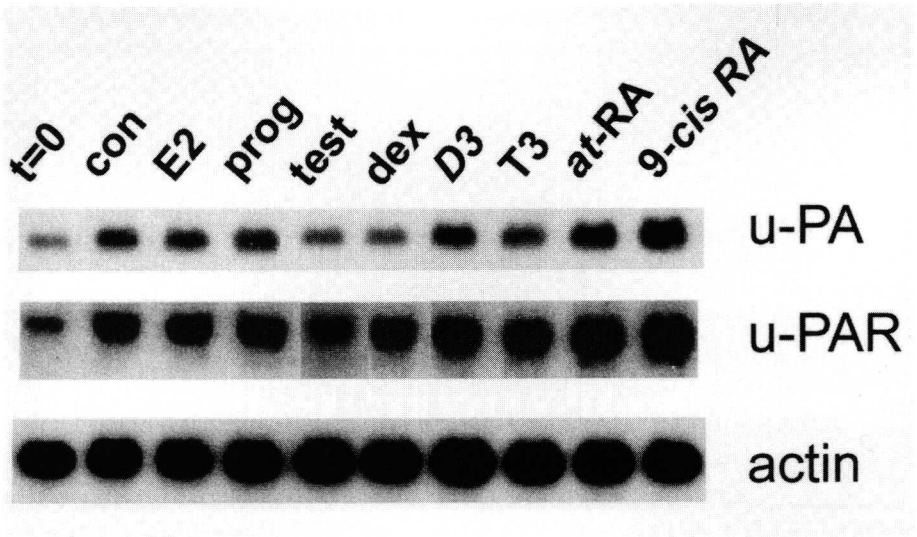

Figure 5. Northern blot analysis of u-PA and u-PAR mRNA of human microvascular endothelial cells cultured in the presence of steroid hormones, retinoids, thyroid hormone or 1,25-dihydroxyvitamin D3. Human microvascular endothelial cells were cultured on gelatin-coated dishes in incubation medium containing 20 ng/mL bFGF and 20 ng/mL TNFα, and 0.01% (vol/vol) DMSO (con) or 1 μmol/L of 17ß-estradiol (E2), progesterone (prog), testosterone (test), dexamethasone (dex), 1,25-dihydroxyvitamin D3 (D3), thyroid hormone (T3), all- *trans* retinoic acid (*at*-RA) or 9-*cis* retinoic acid (9-*cis* RA). After 2 days the media were refreshed. After 4 days RNA was isolated and analyzed (7.5 μg/lane) by Northern blot analysis using 32-[dCTP] labelled probes for u-PA, u-PAR and actin.

Nuclear hormone receptors

To determine whether the effect of testosterone, dexamethasone and *at*-RA on u-PA production and tube formation was mediated by their respective nuclear receptors, experiments in the presence of receptor-specific antagonists were performed. As shown in Figure 6A, the androgen receptor antagonist hydroxyflutamide counteracted the inhibitory effect of R1881. The glucocorticoid receptor antagonist RU486 prevented the suppressing effect of dexamethasone on tube formation (Fig 6B), whereas the RAR antagonist Ro41-5253 completely inhibited the *at*-RA-induced increase in tube formation (Fig 6C). These effects of the receptor-specific antagonists on tube formation were paralleled by similar changes in u-PA production (data not shown). These data strongly suggest a role for nuclear hormone receptors in these hormone-induced changes in tube formation and u-PA production, although the use of antagonists does not always give unequivocal answers (32).

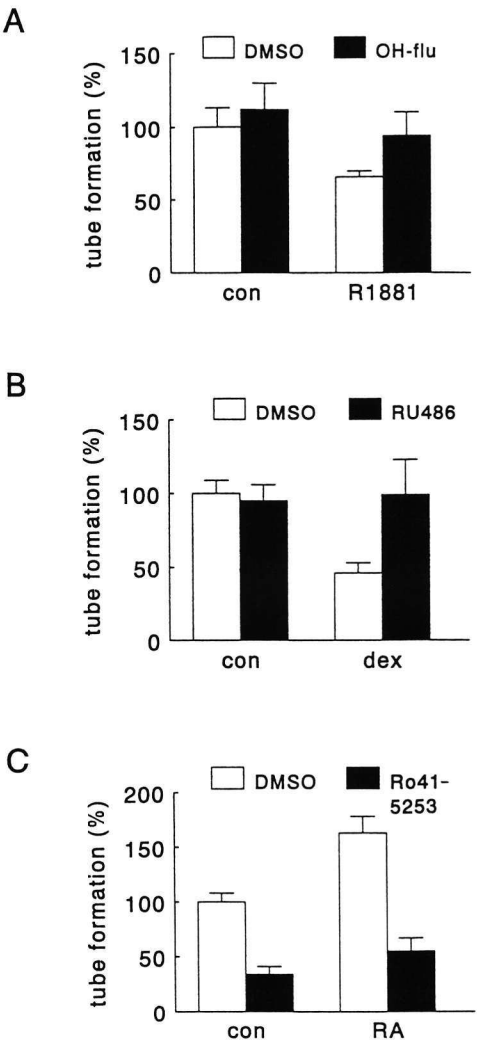

Figure 6. Effects of nuclear receptor antagonists on R1881-, dexamethasone- and *at*-RA- modulated tube formation. Human microvascular endothelial cells were cultured for 8-12 days on three-dimensional fibrin matrices in incubation medium containing 20 ng/mL bFGF and 20 ng/mL TNFα, and to which was added 1 nmol/L R1881 (R1881)(panel A), 10 nmol/L dexamethasone (dex) (panel B) or 10 nmol/L *at*-RA (RA)(panel C). Receptor antagonists (hydroxyflutamide, RU486 or Ro41-5253;final concentration 1 µmol/L for hydroxyflutamide, RU486 and 10 µmol/L for R041-5253) or vehicle (0.1 or 0.01% DMSO) were added 1 hour prior to the hormones.
A: Effect of solvent [DMSO; 0.01 % (vol/vol)] or hydroxyflutamide (OH-flu; 1 (µmol/L) on tube formation of human microvascular endothelial cells in incubation medium alone (con) or supplemented with R1881 (R1881; 1 nmol/L).
B: Effect of solvent [DMSO; 0.01 % (vol/vol)] or RU486 (RU486; 1 µmol/L) on tube formation of human microvascular endothelial cells in incubation medium alone (con) or supplemented with dexamethasone (dex; 10 nmol/L).
C: Effect of solvent [DMSO; 0.1 % (vol/vol)] or Ro41-5253 (Ro41; 10 µmol/L) on tube formation of human microvascular endothelial cells in incubation medium alone (con) or supplemented with all-*trans* retinoic aicd (*at*-RA; 10 nmol/L).
The data represent the average ± standard deviation of 3 experiments performed in duplicate and are expressed as percentages of control values (tube-length/cm^2 in the presence of bFGF and TNFα).

To determine whether the weak effects of some of the hormones tested are related to the low expression levels of the corresponding nuclear receptors, we performed RT-PCR analysis on RNA isolated from hMVEC cultured in incubation medium for 4 days. Indeed, the levels of progesterone receptor mRNA, estrogen receptor α mRNA and estrogen receptor ß mRNA in hMVEC were below the detection limit of our assay (Figs 7A, B and C). Also in cells cultured in the absence of bFGF and TNFα, mRNA for these receptors could not be detected. Only a very weak band for the vitamin D3 receptor could be demonstrated (Fig 7D). Unexpectedly, both thyroid hormone receptor α and ß were clearly expressed (Fig 7E).

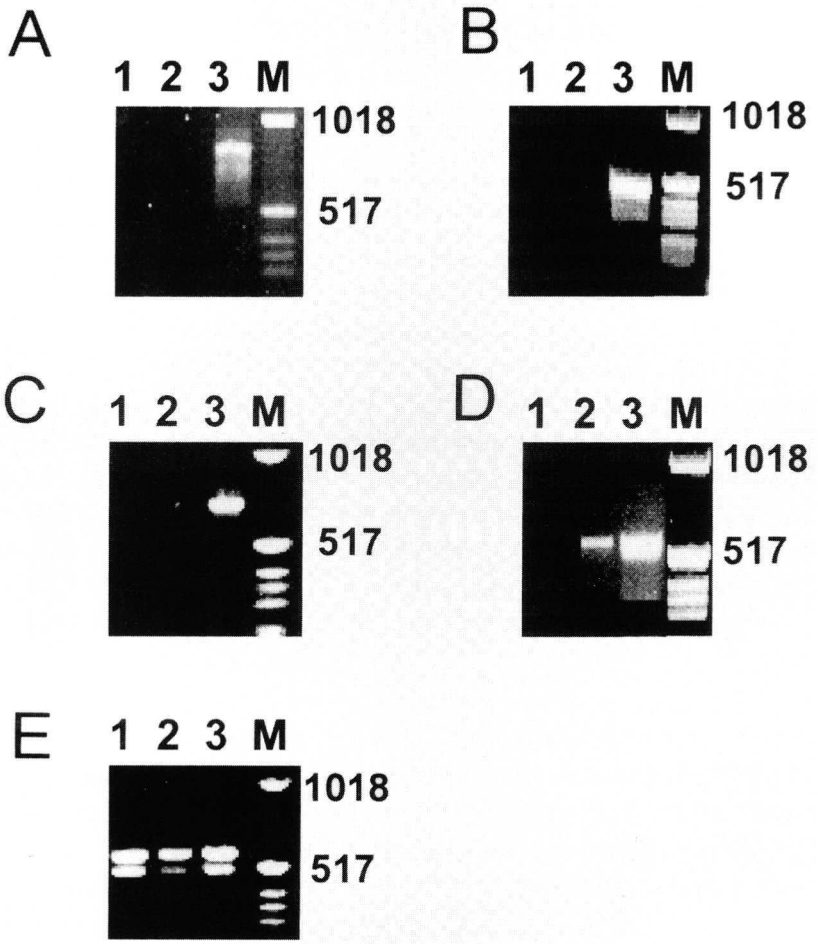

Figure 7. Presence of estrogen receptors α and ß, progesterone receptor, thyroid hormone receptors α and ß and vitamin D receptor mRNA in human microvascular endothelial cells as determined by RT-PCR. Human microvascular endothelial cells were cultured for four days on gelatin-coated dishes in incubation medium containing 20 ng/mL bFGF and 20 ng/mL TNFα or in incubation medium from which bFGF and TNFα was omitted. RNA was isolated from these cells and cDNAs were synthesized using 1 µg total RNA and oligo dT primer as described in Materials and Methods. The cDNAs were amplified with primers for estrogen receptor α (panel A), estrogen receptor ß (panel B), progesterone receptor (panel C), vitamin D receptor (panel D) and thyroid hormone receptors α and ß (panel E) as described in the Materials and Methods section. The expected length of the amplified DNA fragment of the estrogen receptor α is 832 nt, of the estrogen receptor ß 541 nt, of the progesterone receptor 737 nt, of the thyroid hormone receptor α 523 nt, of the thyroid hormone receptor ß 458 nt and of the vitamin D receptor 579 nt. As a positive control for the expression of estrogen receptor α, progesterone receptor, thyroid hormone receptors α and ß and vitamin D receptor mRNA, RNA isolated from MCF7 cells was used. As a positive control for the expression of the estrogen receptor ß mRNA, RNA isolated from the SV-HFO osteoblast cell line was used. Lane 1: incubation medium from which bFGF and TNFα had been omitted, lane 2: incubation medium containing bFGF and TNFα, lane 3: appropiate positive control, M: molecular weight marker.

DISCUSSION

The present study demonstrates that steroid hormones and retinoids can have strong, but different effects on the formation of capillary-like tubular structures by human microvascular endothelial cells cultured on top of a human fibrin matrix in the presence of bFGF and TNFα. We found that testosterone and dexamethasone almost completely block tube formation in this *in vitro* model, while *at*-RA and 9-*cis* RA strongly potentiate the effect of bFGF and TNFα. These compounds are likely to exert their action via their respective nuclear receptors, as demonstrated by the use of receptor-specific antagonists. 17ß-Estradiol, progesterone, thyroid hormone (T3) and 1,25-dihydroxyvitamin D3 showed no or only minor effects on *in vitro* angiogenic activity, which, with the exception of T3, could be related to the absence of significant nuclear receptor expression.

We found that testosterone and dexamethasone decreased, and *at*-RA and 9-*cis* RA increased u-PA mRNA and antigen levels. These alterations in u-PA expression not only parallel, but are also likely to be responsible for the observed changes in angiogenic activity for the following reasons. First, exogenous suppletion of the medium with scu-PA enhances tube formation in our *in vitro* model, whereas quenching of u-PA activity (but not t-PA activity) or u-PA binding to u-PAR by specific antibodies suppresses basal and retinoid-stimulated tube formation. Secondly, addition of scu-PA to testosterone- and dexamethasone-treated hMVEC restores the suppressed angiogenic activity for a substantial part.

The small effect of 17ß-estradiol on tube formation, despite the lack of an effect on u-PA production, may be related to its conversion to 2 methoxyestradiol (2-ME). 2-ME has been shown to inhibit *in vitro* angiogenesis, probably by interfering with tubulin polymerization (15,33). We found 2-ME to inhibit tube formation in our model without affecting u-PA expression. Inasmuch the absence of a direct effect of 17ß-estradiol on angiogenesis is related to the male origin of the endothelial cells (viz. human foreskin) is not clear at present. For a proper assessment of the role of sex steroids in angiogenesis, it may be relevant to extend our present experiments to endothelial cells of female origin.

The mechanism by which the steroid hormones and retinoids alter the TNFα-stimulated u-PA expression in hMVEC is not known, but may be related to activation of the NFκB/Rel system by TNFα (34, 35). In unstimulated endothelial cells, NFκB/Rel family complexes are retained in the cytoplasm by the binding of inhibitory proteins, including IκBα. Endothelial activation evokes dissociation of IκBα and allows translocation of the transcription factors to the nucleus. Two functional NFκB elements at -1580 and -1865 bp have been identified in the human u-PA promoter (36). We have found, using a recombinant adenovirus expressing IκBα, that overexpressed IκBα inhibits cytokine-stimulated NFκB activation and u-PA expression in human umbilical vein endothelial cells (Kooistra and De Martin, unpublished data). The dexamethasone-binding glucocorticoid receptor can directly or indirectly bind and inactivate the

NFκB transcription factor, analogous to the mechanism responsible for the negative interaction between the glucocorticoid receptor and the AP-1 transcription complex (37, 38, 39). More recent studies have demonstrated that dexamethasone stimulates the synthesis of the IκBα gene which may then result in trapping of activated NFκB in inactive cytoplasmic complexes (40, 41), although such a mechanism is not yet generally accepted (42). Testosterone, whose androgen receptor belongs to the same subclass of nuclear receptors as the glucocorticoid receptor, might also act by androgen receptor-mediated inhibition of NFκB-mediated gene transcription (43). Additionally or alternatively, testosterone may act via maintaining IκB levels, as suggested by Keller et al. for the repressive effect of androgens on the expression of interleukin-6 (44).

Our finding that *at*-RA and 9-*cis* RA by themselves only slightly increase u-PA expression, but strongly up-regulate the TNFα-induced u-PA synthesis, resembles the results described by Harant et al. for the activation of IL-8 gene transcription in the human melanoma cell line A3 (45). It was shown that stimulation with *at*-RA and TNFα resulted in enhanced NFκB binding compared to that induced by TNFα alone and also resulted in changes in the composition of the NFκB complexes bound to the IL-8 NFκB site.

Although the present study underlines the importance of u-PA in determining tube formation in a fibrin matrix, we cannot exclude the possibility that the steroids and retinoids alter expression of other relevant parameters as well. One indication is that *at*-RA and 9-*cis* RA increase and testosterone decreases u-PAR mRNA levels in our model system. The u-PAR provids the cell a mechanism for localized proteolysis (46,47), which may be helpful for a tightly controlled and restricted proteolysis, both in time and in space, which is essential in the angiogenic process. Moreover, u-PAR can act as an adhesion receptor for vitronectin (48), thereby providing the cell an adhesive interaction with components of the extracellular matrix, which is another important requirement for angiogenesis to occur. Other possible regulators of the angiogenic process which are susceptible to regulation by steroids and retinoids include thrombospondin, integrins and matrix-degrading metalloproteases (MMPs). Thrombospondin-1, shown to suppress the angiogenic response *in vivo* and *in vitro*, is regulated by progesterone in the human endometrium (49-51). Since the glucocorticoid and progesterone receptor share the same nucleotide binding sequence, dexamethasone could also enhance secretion of thrombospondin-1 in hMVEC. Similarly, *at*-RA has been shown to induce the expression of integrins like $\alpha_v\beta_3$ and β_4 (52, 53). These integrins are cell-membrane proteins which connect the cell cytoskeleton with matrix proteins such as fibrin, can transduce signals into the cell and have been implicated in angiogenic processes (54). The expression of members of the MMP family, which are involved in the breakdown of the extracellular matrix, can be inhibited by glucocorticoids and retinoids (55-57). This repression has been shown to be mediated by the AP-1 binding site, present in the promoters of these genes (58,59).

Changes in effecters other than u-PA may be particularly relevant in angiogenesis models based on non-fibrin matrices like collagen or mixed matrices, and may also explain some of the

seemingly conflictory results between different studies. For example, Pepper et al. showed that *at*-RA inhibited tube formation in their *in vitro* model, using bovine endothelial cells cultured on a collagen matrix, despite the *at*-RA-induced increase in u-PA production (60). Because collagen type-1 matrices are degraded effectively only by MMPs, these results are probably due to a reduced production of MMPs in the presence of *at*-RA. Retinoids were also shown to be effective inhibitors of angiogenesis in the CAM assay (9,10). However, this model system reflects embryonic angiogenesis, where, to our knowledge the formation of blood vessels is independent of the presence of inflammatory mediators and where the composition of the extracellular matrix is probably very distinct from the matrix present in for example wounds and tumors.

Our *in vitro* model system reflects pathological angiogenesis in man in the presence of fibrin and inflammatory mediators. Our results are therefore probably most relevant for angiogenic recanalization of a fibrin clot, neovascularization in tumor stroma and the formation of new blood vessels in the temporary fibrin matrix present at sites of chronic inflammation. The different effects of the diverse hormones on angiogenesis in this model system underline the importance of the microenvironment in the angiogenic process. Our findings provide insight into the mechanism whereby normally circulating hormones influence the angiogenic activity of endothelial cells and are relevant for developing tools to influence pathological angiogenesis.

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CHAPTER 7

GENERAL CONCLUSIONS

CONTENTS

In this chapter some specific findings described in this thesis will be discussed in a broader context. Our finding that the induction of tissue-type plasminogen activator (t-PA) gene expression by retinoic acid (RA) in human endothelial cells is mediated via specific retinoic acid receptor (RAR) subtypes raises questions about the significance of the various RAR (and retinoid X receptor (RXR)) subtypes in gene transcription in general. Do various RAR/RXR subtypes preferentially control the transcription of different subsets of RA-responsive genes? In this context attention is also paid to the possible receptor-subtype specific actions of the estrogen receptor (ER) and the consequences of such a specificity in normal physiology, but also for the development of synthetic estrogens with more specific effects.

The identification of an active all-*trans*-retinoic acid (*at*-RA) metabolic system in human endothelial cells, involving auto-inducible cytochrome P-450 enzymes, revealed the presence of a hitherto neglected property of the endothelium. This capacity of endothelial cells to perform cytochrome P-450-mediated metabolic activities may have consequences for RA-treated diseases such as acute promyelocytic leukemia, and for RA metabolism in general.

Finally, our finding that steroid hormones and retinoids (Vitamin A and derivatives thereof) can have a profound effect on tube formation in an *in vitro* angiogenesis model, suggests that processes such as angiogenesis can be effected by normally circulating hormones. Some of the consequences of these effects will be elaborated on.

7.1 RECEPTOR SUBTYPE SPECIFICITY

7.1.1 Retinoic acid receptors

In this thesis we showed that a specific RAR subtype, RARß2, which itself is induced via RARα, is the ultimate mediator of RA-induced t-PA expression in cultured human endothelial cells. These results are a clear example of receptor-subtype specificity among the RARs. Leid et al. (1992) already suggested that each RAR and RXR subtype and isoform may perform unique functions. This concept was based on the following observations: (1) the amino acid sequences of receptor subtypes are highly conserved among different species; (2) each of the RAR/RXR subtypes exhibits a spatially and temporally specific pattern of expression during embryonic development. In time, experimental support for this hypothesis has been obtained, although several studies also demonstrated the presence of receptor redundancy. For example, some *in vitro* studies showed that distinct RAR and RXR receptor subtypes and isoforms exhibit different transactivation activities on naturally occurring and synthetic (DR-1 and DR-5) retinoic acid-response element (RARE)-containing reporter genes (Nagpal et al., 1992; LaVista-Picard et al., 1996). However, other *in vitro* studies revealed no substantial differences between the different receptor subtypes in transactivating RARE-containing reporter genes (Durand et al., 1992; Robertson et al., 1992; Pratt et al., 1993; Bulens et al., 1996). A common denominator of these aforementioned studies is that they

were performed with cultured cells cotransfected with vectors overexpressing receptors and recombinant chimeric reporter genes. This situation is far from physiological in terms of both receptor concentration and chromatin environment, and is not suitable to give the final answer to the question of receptor-subtype specificity.

To circumvent (part of) the above-mentioned drawbacks of transfection studies, much effort has been made recently to investigate the specificity of RAR and RXR subtypes by using receptor-subtype specific synthetic ligands and by using RAR and/or RXR subtype "knock-out" cell lines and mice (Lohnes et al., 1993; Lufkin et al 1993; Luo et al., 1993; Boylan et al., 1993; Boylan et al., 1995; Roy et al., 1995; Taneja et al., 1995, 1996; Clifford et al., 1996; Krężel et al., 1996; Chiba et al., 1997). The use of RAR subtype-specific synthetic ligands demonstrated that specific RAR subtypes activate different RA-responsive genes to different extents (Roy et al., 1995; Taneja et al., 1996; Chiba et al., 1997). For instance, an RARγ-selective retinoid induced the expression of the Stra8 gene much more efficiently than did an RARα agonist, whereas an RARβ agonist was completely ineffective (Chiba et al., 1997). Also, studies using cells lacking one RAR or RXR subtype or a combination of one RAR and one RXR subtype, so-called "double knock outs" (Boylan et al., 1993, 1995; Taneja et al., 1995, 1996; Roy et al., 1995; Clifford et al., 1996; Chiba et al., 1997), further support the idea that different RAR/RXR subtypes possess distinct biological activity. For instance, the disruption of the RARγ gene in F9 cells resulted in a lowered RA induction of several RA-responsive genes, including Hoxa1, Gata4 and TCF2 (Taneja et al., 1995). Another study, using RAR/RXR-subtype double mutant F9 cells, demonstrated that the heterodimer RXRα/RARγ was the most efficient activator of Hoxa-1 expression, with other heterodimer combinations being less efficient or even unable to modulate Hoxa-1 expression (Chiba et al., 1997).

Although the above studies on receptor-subtype specificity indicate that different RAR and/or RXR subtypes exert specific effects on the expression of particular sets of RA-responsive genes, it also became apparent from other studies that one receptor subtype can substitute for another one (i.e. show redundancy) in inducing gene expression, albeit not always with the same efficiency (Taneja et al., 1995, 1996; Chiba et al., 1997). For instance, in a study of Chiba et al. (1997), using single or double RAR- or RXR-subtype knock-out F9 cells, several RA target genes are highly efficiently induced by more than one RAR/RXR receptor subtype combination (Chiba et al., 1997). Furthermore, receptor redundancy appeared to be dependent on the promoter context of the response element and on the cell type. For instance, an agonist for RARβ in combination with a pan-RXR agonist (capable of activating all three RXR subtypes) was able to induce Hoxa-1 and RARß2 expression in P19 cells. In F9 cells on the other hand, the RARß agonist in combination with the pan-RXR agonist was unable to induce these genes (Taneja et al., 1996), despite the presence of RARß in these cells (Martin et al., 1990). These cell type-dependent differences in gene induction

via specific receptor subtypes could be due to the presence or absence of receptor-subtype specific cofactors for transcription activation, and underline the importance of physiological context in studies on receptor specificity and/or redundancy.

An important finding from studies with RAR subtype null mutant cell lines is that gene targeting itself can induce functional redundancy between receptor subtypes (Taneja et al., 1996; Chiba et al., 1997). The presence of a given receptor subtype can hinder the activity of another subtype, and inactivation of such a subtype will consequently abolish this hindrance. For instance, a RARα synthetic ligand induces Hox1-a and RARß2 transcripts efficiently in a RARγ$^{-/-}$ F9 cell line, but only to a small extent in the wild type cell line, indicating that the presence of RARγ prevents RARα to mediate efficiently the induction of these genes. This arteficial introduction of receptor redundancy in "knock out" cell lines implies that results obtained in this kind of studies should be interpreted with caution.

In disagreement with the presence of receptor-subtype specific induction of several RA target genes as found in *in vitro* studies are also the phenotypes of "null mutant" mice for single RAR or RXR subtype genes. Unexpectedly, these mice show no or only mild phenotypes (Lohnes et al., 1993; Lufkin et al., 1993; Luo et al., 1993; Krężel et al., 1996), which results argue for functional redundancy among the different RAR subtypes. However, the presence of a genetic "adaption" in the absence of a particular gene product, as was found also for "knock-out" cell lines, may form an alternative explanation for the phenotypes in these mice. In support herewith, the phenotypes of various combinations of retinoid receptor-subtype knock outs are much more severe than of single mutants (Mendelsohn et al., 1994; Kastner et al., 1995) and are more in agreement with the presence of receptor-subtype specificity.

What can we conclude from all these studies on receptor sybtype specificity ? First, the findings suggest that in a particular physiological situation a specific RAR/RXR subtype combination will preferentially be involved, although examples exist in which all three RAR subtypes possess the capability to mediate the induction of an RA target gene. The composition of the receptor subtype combination will be primarily determined by the response element of the target gene and its promoter context. In addition, cell-specific expression of cofactors and relative concentrations of the different receptor sybtypes are important, sometimes even crucial determinants of effective receptor sybtype combinations. Especially now it is known that receptor subtypes are able to antagonize each other, this latter factor (i.e. relative receptor subtype concentrations) may be of importance.

A second conclusion is that, because of the importance of the cellular environment, it is necessary to study the phenomenon of receptor subtype specificity as close as possible in its physiological context. The results obtained with cell lines or mice in which redundancy is arteficially created by introducing a non-physiological environment via gene disruption, clearly illustrate this. In this respect the development of RAR-subtype specific ligands will

provide useful tools for determining which receptor subtype may specifically mediate a retinoid event under physiological conditions.

7.2.1 Estrogen receptors

The recent identification of a second estrogen receptor, ERβ (Kuiper et al., 1996; Mosselman et al., 1996), raises also the possibility of receptor-subtype specific functions for estrogen receptors. To date, much research is being performed to identify the possible specific functions of ERα and ERß. The *in vivo* distribution of both receptors is partially distinct and there is a high degree of interspecies conservation among each ER subtype (Kuiper et al., 1997), both suggestive for receptor-subtype specific functions. Receptor-subtype specificity is further supported by the recent *in vitro* finding that estrogen and diethylstilbestrol are able to induce activator protein-1 (AP-1) mediated gene expression via ERα but not via ERß (Paech et al., 1997). Antiestrogens, including tamoxifen, are able to stimulate AP-1-mediated transcription via both receptor types. The authors suspect that in the body both estrogen receptors produce different effects as well, depending on the type of cell where they are located and the identity of the ligand. A study by Iafrati et al. (1997) also offered some indications that there may well be specific functions for ERß. They showed that estradiol inhibits the response to vascuclar injury in ERα "knock-out" mice to the same extent as in control mice and they also showed the expression of ERß mRNA in the vasculature. The differences in activity of the two ERs, together with their different distributions in the body, may be at the base of estrogen's broad spectrum of activities, and may help explain some of the mysteries of estrogen action. For example, the lack of the "classic" estrogen receptor ERα in the prostate epithelium (a tissue that is very sensitive to the action of estradiol (Kruithof-Dekker et al., 1996)) and the differential effects of the estrogen antagonist, tamoxifen, in different tissues (Katzenellenbogen et al., 1995) are but two examples of the many unexplained effects of this hormone.

The development of synthetic estrogens and anti-estrogens based on the existence of two ERs would have more specific effects than the drugs currently in use because they interact with one receptor and not the other. This could be relevant in the design of an estrogen that could, for example, protect postmenopausal women against cardiovascular problems without raising their risk of developing breast or uterine cancers, as current supplements do. Besides a reduction of unwanted side-effects, it will also be interesting to learn whether such new generation drugs also show the broad spectrum of effects as current compositions of estrogen replacement therapy (ERT) do. Presently, ERT is thought to exert its beneficial effects through direct estrogen action on blood vessels and by decreasing LDL cholesterol and increasing HDL cholesterol levels (Kushwaha et al., 1981; Fahrat et al., 1996) In addition, our findings in Chapter 5 indicate that estrogen treatment can have profound effects on the clearance rate of t-PA via induction of mannose receptor expression in the liver. At present, it

is unknown whether these effects on hepatic mannose receptor expression are mediated via ERα or ERß.

More specific estrogens could also be of benefit to protect against Alzheimer's disease and osteoporosis, while new analogues of tamoxifen may counteract estrogen-promoted breast cancer without increasing the occurence of uterine cancer.

7.2 CYTOCHROME P-450 MEDIATED RETINOIC ACID METABOLISM IN ENDOTHELIAL CELLS

Our studies on the metabolism of *at*-RA in human endothelial cells as described in Chapter 4, demonstrate that these cells possess a very active, cytochrome P-450- mediated *at*-RA metabolizing capacity, comparable to that of human hepatocytes. On the other hand, we noted some interesting differences in RA metabolism between both cell types. In human endothelial cells, but not in human hepatocytes, *at*-RA induces it own metabolism. Furthermore, different cytochrome P-450 inhibitors decreased *at*-RA metabolism in both cell types to different extents: while in endothelial cells liarozole is a much more potent inhibitor of *at*-RA metabolism than ketoconazole, both cytochrome P-450 inhibitors are equipotent in retarding *at*-RA metabolism in hepatocytes.

These *in vitro* findings on RA metabolism may provide new insights into RA metabolism *in vivo*. The liver is an established site for RA metabolism (Roberts et al., 1979; Leo et al., 1989; Roberts et al., 1992). However, our finding that endothelial cells actively metabolize *at*-RA suggests the presence of a great extrahepatic capacity for RA metabolism. Especially because the endothelium forms a large surface area (estimations range from 720-1500 m^2) to which plasma *at*-RA is continuously exposed (Wolinsky, 1980; Schmidt, 1989), endothelial cells may participate substantially in the metabolism of *at*-RA. Support for the existence of substantial extrahepatic *at*-RA metabolism also comes from *in vivo* experiments in rats, in which liarozole proved to be a much more potent inhibitor than ketoconazole (Wauwe et al., 1990). These results substantiate our hypothesis that extrahepatic tissues, including the endothelium, may significantly contribute to *at*-RA metabolism *in vivo* (Wauwe et al., 1990). In addition to the endothelium, also other extrahepatic tissues are found to be able to degrade *at*-RA, including testis, skin and lung (Leo et al., 1989; Robert et al., 1989; Duell et al., 1992; Martini and Murray, 1993; Fiorella and Napoli, 1994; White et al., 1996). However, the relative contribution of the different extrahepatic tissues to *at*-RA metabolism is not exactly known. Considering the pharmacological use of RA and the problems related to its use (see below), more knowledge on the contribution of extrahepatic tissues on RA metabolism is required.

The cytochrome P-450-mediated metabolizing capacity of endothelial cells is not

restricted to the metabolism of retinoids. Several studies have shown that endothelial cells are also able to metabolically activate protoxicants (Brittebo, 1994; Farin et al., 1994) and generate vasoactive metabolites (reviewed by Harder et al., 1995). Undoubtly, other endothelial-specific, cytochrome P-450-dependent metabolic processes will be identified in the future. Especially due to the continuous exposure of endothelial cells to compounds present in plasma, endothelial cells will be able to contribute substantially to cytochrome P-450-mediated reactions, such as detoxifying xenobiotics or activation of procarcinogens, which processes are now believed to be primarily mediated by the liver. As a consequence, assessment of the role of cytochrome P-450 enzymes in, for instance, drug metabolism or assessment of toxicity of chemicals should not be limited to studies with hepatocytes, but should also include endothelial cells.

Another interesting finding from our RA metabolism studies is the auto-inducibility of *at*-RA metabolism in human endothelial cells (Chapter 4). Such an inducible *at*-RA metabolism may be of clinical significance, for instance in the treatment of acute promyelocytic leukemia (APL) by *at*-RA (Huang et al., 1988). In this disease, *at*-RA is used to induce remission (Warrell et al., 1991), but most patients relapse and develop *at*-RA-resistant disease (Castaigne et al., 1990; Warrell et al., 1991). One explanation for this RA resistance is the induction of *at*-RA metabolic activity during the treatment with *at*-RA, resulting in decreased *at*-RA plasma levels (Muindi et al., 1992). The specific cytochrome P-450 isoform(s) involved in (RA- induced) RA metabolism and consequently the possibility to specifically interfere in this *at*-RA-induced RA metabolism, were unknown for a long time. However, recently a human RA metabolizing cytochrome P-450 isoform was identified, hP450RAI (White et al., 1997). This cytochrome P-450 is expressed in a number of human cell lines and in part of these cell lines its expression is induced by *at*-RA, suggesting a cell-specific regulation (White et al., 1997). The expression of hP450RAI in human endothelial cells was not investigated, but this isoform is unlikely to be the enzyme responsible for the rapid auto-induced *at*-RA degradation as found in our studies. Argueing against this possibility is our finding that endothelial cells metabolize 9-*cis* RA very slowly, whereas 9-*cis* RA was shown to be a good substrate for hP450RAI. A better candidate for endothelial cell RA metabolism is the RA-metabolizing enzyme found in human skin, where a cytochrome P-450 isoform hydroxylizes *at*-RA in a RA-inducible way, but not 9-*cis* RA (Duell et al., 1996).

In aggregate, these results indicate the presence of several tissue- specific (RA-inducible) cytochrome P-450 isoforms mediating RA metabolism. Further knowledge on these tissue-specific isoforms and their contribution to the RA-induced metabolism would facilitate the design of specific inhibitors to counteract the increased RA metabolism in APL patients. In general, such knowledge would lead to a better understanding of the role of RA metabolism in regulating RA levels under normal conditions and in disease.

7.3 MODULATORY EFFECTS OF HORMONES ON U-PA-MEDIATED PROCESSES SUCH AS ANGIOGENESIS

In Chapter 6 it is shown that testosterone and dexamethasone almost completely block TNFα and bFGF-induced tube formation in an *in vitro* angiogenesis model, whereas RA strongly enhances the bFGF and TNFα -induced tube formation. Furthermore, we demonstrated that the effects of these hormones on *in vitro* angiogenesis could be explained by their effects on u-PA synthesis and are mediated via their respective nuclear receptors. Dexamethasone and testosterone inhibited bFGF and TNFα-induced u-PA synthesis, whereas RA enhanced u-PA expression. The absence or only minor effects of 17ß-estradiol, progesterone, thyroid hormone, and 1,25-dihydroxyvitamin D3 on *in vitro* angiogenesis could, with the exception of thyroid hormone, be related to the absence of significant expression of the corresponding nuclear receptors.

These results nicely illustrate that hormones are able to influence the effects of growth factors and/or inflammatory mediators on u-PA gene expression. More generally, these modulating effects of hormones on angiogenesis via u-PA expression imply that systemically present hormones are able to affect local u-PA-mediated processes involving growth factors and/ or inflammatory mediators. Another example of such a local u-PA-involved process that may be affected by systemic hormones is wound healing in the skin. This complex process involves formation of a matrix rich in fibrin and fibronectin in the wound field, infiltration of inflammatory cells, proliferation and migration of epidermal keratinocytes at the wound edges, formation of granulation tissue containing newly developed vessels, migration of inflammatory cells and fibroblasts, and wound contraction (Clark and Henson, 1988; Donaldson and Mahan, 1988). During several processes in wound healing u-PA is believed to play a role, including migration of keratinocytes, macrophages, granulocytes and fibroblasts, and angiogenesis (Heiple and Ossowski, 1986; Rømer et al., 1991, 1993; Vassalli et al., 1992). Many of these u-PA-mediated processes are induced by locally produced growth factors and inflammatory mediators and are potentially a target for hormone modulation. Indeed, glucocorticoids are well-known inhibitors of wound healing (Beer et al., 1997). Although their anti-inflammatory potential could explain their anti-wound healing effects, inhibition of locally induced u-PA expression by these steroids is also able to contribute to impaired wound healing. Interestingly, RA and its precursor vitamin A ameliorate (impaired) wound healing in animals and in humans (Seifter et al., 1981; Levenson et al., 1984; Weinzweig et al, 1990; Popp et al., 1995). Moreover, vitamin A seems to effectively counteract the deleterious effects of glucocorticoids on wound healing in both animals and humans (Hunt et al., 1969; Phillips et al., 1992). Wound healing is a multistep process involving the interplay of a lot of different cell types, and retinoids may affect any of these steps. However, the positive effects of retinoids on u-PA expression and herewith related

processes may be one of the mechanisms of action.

Another example of an u-PA mediated process that could be affected by hormones is the proliferation and migration of smooth muscle cells (SMC) during atherosclerosis. In plaques elevated concentrations of several growth factors and cytokines have been demonstrated, which are capable of inducing SMC migration and proliferation (Clinton and Libby, 1992; Raines and Ross, 1996). Support for a modulatory role of hormones in this process comes from an *in vitro* study with human aortic SMC. This study demonstrates that 17-β estradiol inhibits the proliferation and platelet-derived growth factor-induced migration of SMC (Suzuki et al., 1996).

Steroid hormones and retinoids also modulate the growth factor- and/or cytokine-mediated induction of genes other than u-PA, for example the interleukin-8 or nitric oxide synthase II genes (Mukaida et al., 1994; Harant et al., 1996; Kleinert et al., 1996). These findings underline that systemic hormones are able to modulate the effects of locally occurring processes. So, besides their direct role in a variety of physiological procesess, steroid hormones and retinoids are also potent modulators of local growth factor/ cytokine-mediated phenomena.

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SUMMARY

Chapter 1 introduces the plasminogen activation system, a highly regulated enzymatic cascade for obtaining extracellular proteolysis. Two types of plasminogen activators exist, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Both activators are able to convert the inactive proenzyme plasminogen into the broad spectrum protease plasmin, which can degrade fibrin as well as extracellular matrix proteins. Since many different biological processes are dependent on the plasminogen activation system, including proteolytic degradation of fibrin clots (fibrinolysis) and the formation of new blood vessels (angiogenesis), its activity needs to be highly regulated. Various cytokines, growth factors, hormones and vasoactive compounds have been reported to modulate the activity of the system. In this thesis the effects of steroid hormones and retinoids (vitamin A and derivatives thereof) on fibrinolysis and angiogenesis have been studied. In particular we have concentrated on the mechanism(s) via which hormones exert their action. We have investigated the role of nuclear receptors and metabolism in the retinoid-stimulated expression of t-PA in cultured human endothelial cells, the effects of estrogens on hepatic clearance of t-PA, and the molecular mechanism by which hormones and retinoids modulate tube formation in an *in vitro* angiogenesis model.

The studies described in Chapter 2 were performed to identify the retinoic acid receptors (RARs) and retinoid-X-receptors (RXRs) that are involved in the induction of t-PA by retinoids in human umbilical vein endothelial cells (HUVEC). Northern blot analysis demonstrated the presence of mRNA transcripts for RAR subtype α (RARα), RXRα and, at much lower levels, RARß, RARγ and RXRß. Upon the addition of retinoic acid (RA) the mRNAs for RARß and RXRα were markedly induced. By the use of retinoid analogues that bind preferentially to one of the RAR or RXR subtypes, we showed RARα to be involved in the retinoid-induced t-PA expression in HUVEC. The conclusion that RARα is mediating the retinoid-induced t-PA gene expression was further strengthened by experiments in which selective blocking of RARα with a specific RARα antagonist, Ro 41-5253, strongly reduced RA-stimulated t-PA expression in HUVEC.

The induction of t-PA by RA in HUVEC is a rather slow process. This prompted us to investigate this process in more detail, in particular whether or not RARα induces t-PA expression in a direct manner (Chapter 3). The complete inhibition of the induction of t-PA by RA in the presence of the protein synthesis inhibitor cycloheximide (CHX) indicated that RARα does not induce t-PA synthesis directly, but indirectly via another protein. Since the induction of RARß by RA exactly preceded that of t-PA, we investigated the possibility that this RAR subtype is the final mediator of t-PA induction. We found that HUVEC with elevated RARß mRNA levels -as a result of pre-treatment of the cells with RA and/or CHX- showed an undelayed increase in t-PA expression by RA. An essential role of RARβ was

further proven by performing antisense experiments. An antisense oligodeoxynucleotide against the translation initiation site of RARβ2 mRNA greatly suppressed the induction of t-PA by RA. An antisense oligodeoxynucleotide blocking RARβ4 was ineffective. Taken together, these results point to a two-step mechanism in which RARβ2 is first induced via RARα and then RARβ2 mediates the induction of t-PA.

Because rather high RA concentrations are necessary to induce t-PA synthesis in cultured human endothelial cells as compared to the *in vivo* situation, we studied the metabolism of all-*trans* retinoic acid (*at*-RA) and 9-*cis* retinoic acid (9-*cis* RA) in HUVEC and, for comparison, in the human hepatoma cell line HepG2 and primary cultures of human hepatocytes (Chapter 4). Our results demonstrate the following: (1) human endothelial cells rapidly metabolize *at*-RA, at a comparable rate as human hepatocytes, and this metabolic capacity of endothelial cells, but not that of hepatocytes, is inducible by *at*-RA; (2) in contrast to *at*-RA, 9-*cis* RA is metabolized very slowly by endothelial cells, whereas hepatocytes metabolize 9-*cis* RA even at a higher rate than they metabolize *at*-RA; (3) hepatocytes, but not endothelial cells, are able to isomerize *at*-RA to 9-*cis* RA and vice verca; (4) in both cell types, the cytochrome P450 inhibitors liarozole and ketoconazole are able to inhibit the metabolism of *at*-RA, albeit to different extents. In the presence of the most potent RA metabolism inhibitor in endothelial cells, liarozole, at least 10-fold lower *at*-RA concentrations are required than in the absence of the inhibitor to obtain the same induction of t-PA. These results demonstrate that rapid metabolism of *at*-RA explains the relatively high concentration of *at*-RA required to induce t-PA *in vitro* in cultured endothelial cells.

In Chapter 5 we tested the hypothesis that estrogens lower plasma levels of t-PA by increasing its clearance from the bloodstream. We demonstrated that ethinylestradiol administration increases the clearance of exogenous recombinant human t-PA in mice and that of endogenous, bradykinin-released t-PA in rats. Two distinct t-PA clearance mechanisms exist, the mannose receptor system and via the low-density lipoprotein receptor-related protein (LRP) system. Our results indicate that upregulation of the mannose receptor by ethinylestradiol is most likely responsible for the increased t-PA clearance. First, inhibition of LRP either by injection of receptor-associated protein fused to glutathione S-transferase (GST-RAP) or by overexpression of RAP by adenoviral gene transduction did not eliminate the differene in clearance between control and ethinylestradiol-treated mice, whereas in the presence of a mannose receptor antagonist, mannan, t-PA clearance in control and ethinylestradiol-treated mice became identical. Secondly, ethinylestradiol-treatment of mice induced a 6-fold increase in liver mannose receptor mRNA expression, while the amount of LRP mRNA in the ethinylestradiol-treated animals did not differ from that in control animals. These findings were confirmed at the protein level by Western blotting and ligand blotting, respectively. In conclusion, the results show that ethinylestradiol treatment results in increased plasma clearance of t-PA via a strong induction of the mannose receptor,

and provide an explanation for the inverse relationship between estrogen status and plasma t-PA concentrations.

In Chapter 6, the effects of various hormones on the angiogenic process were studied, using an *in vitro* angiogenesis model in which human microvascular endothelial cells were cultured on top of a human fibrin matrix in the presence of basic fibroblast growth factor (bFGF) and tumour necrosis factor alpha (TNFα). In this model testosterone and dexamethasone almost completely blocked tube formation, whereas RA strongly enhanced the bFGF- and TNFα -induced tube formation. The effects of these hormones were parallelled by similar changes in u-PA mRNA and protein levels: testosterone and dexamethasone decreased and RA increased u-PA expression. Results of studies in which u-PA was added exogenously or in which u-PA activity was quenched by antibodies, strongly suggest that the hormone-induced changes in u-PA levels not only parallel, but also are responsible for the observed changes in angiogenic acitivity. The compounds are likely to exert their action via their respective nuclear receptors, as demonstrated by the use of receptor-specific antagonists. The absence or only minor effects of 17ß- estradiol, progesterone, thyroid hormone, and 1,25-dihydroxyvitamin D3 on *in vitro* angiogenesis could, with the exception of thyroid hormone, be related to the absence of significant nuclear receptor expression.

In Chapter 7, general conclusions from the studies described in this thesis are drawn, and its consequences discussed in a broader context.

SAMENVATTING

Het plasminogeen activatie systeem speelt in dit proefschrift een centrale rol. Dit systeem bestaat uit een enzymatische cascade met als uiteindelijk werkzame component het enzym plasmine. Plasmine is een enzym dat verschillende eiwitten kan afbreken zoals fibrine, de "draadstruktuur" van bloedstolsels, en verschillende eiwitten die een onderdeel uitmaken van de extracellulaire matrix. Plasmine komt normaal in het lichaam voor in een inactieve vorm, plasminogeen, en wordt pas werkzaam na activatie door plasminogeen activatoren. Er bestaan twee verschillende plasminogeen activatoren: weefsel-type plasminogeen activator (t-PA) en urokinase-type plasminogeen activator (u-PA). Het plasminogeen activatie systeem is bij veel verschillende biologische processen betrokken, onder andere bij het oplossen van bloedstolsels (fibrinolyse), het uitzaaien van tumorcellen, het herstel van wonden en de vorming van nieuwe bloedvaten (angiogenese), en derhalve wordt de activiteit van dit systeem nauw gereguleerd. Verschillende componenten -inclusief groeifactoren, cytokines (ontstekingsmediatoren) en hormonen- kunnen de synthese van de verschillende componenten van het plasminogeen activatie systeem, en daarmee de activiteit, moduleren. In het onderzoek dat beschreven staat in dit proefschrift, is het effect van retinoiden (vitamine A en derivaten hiervan) en steroid hormonen (zoals bijvoorbeeld oestrogenen) op twee plasmine-afhankelijke processen bestudeerd, fibrinolyse en angiogenese. In het bijzonder hebben we ons geconcentreerd op het werkingmechanisme van retinoiden en steroid hormonen. Retinoiden zoals vitamine A zuur (retinoic acid, RA) verhogen de productie van t-PA door gekweekte humane endotheelcellen, de binnenste laag cellen van bloedvaten. Wij hebben bestudeerd wat de rol is van nucleaire hormoon receptoren en het metabolisme van RA in deze verhoogde t-PA productie. Verder is het effect van oestrogenen op de klaring van t-PA door de lever onderzocht. Tenslotte is met een *in vitro* model bestudeerd via welk moleculair mechanisme hormonen en retinoiden de vorming van bloedvaten beïnvloeden.

De studies beschreven in hoofdstuk 2 zijn uitgevoerd om te onderzoeken welke RA receptoren (RARs) en retinoid X receptoren (RXRs) de inductie van t-PA door RA mediëren in gekweekte humane endotheelcellen geïsoleerd uit navelstrengvenen. Met behulp van zogenaamde Northern blot analyse werd in deze endotheelcellen de aanwezigheid aangetoond van mRNA voor het RAR subtype α (RARα), RXRα en, in kleinere hoeveelheden, RARβ, RARγ en RXR. Incubatie van de endotheelcellen met RA verhoogde de mRNA niveau's van RARβ en RXRα. Door gebruik te maken van synthetische liganden die bij voorkeur binden aan een bepaald receptor subtype, hebben we aangetoond dat RARα betrokken is bij de inductie van t-PA door RA in endotheelcellen. De conclusie dat RARα de inductie van t-PA medieert werd versterkt door experimenten waarin een selectieve remming van RARα door een specifieke RARα antagonist, Ro 41-5253, de RA gestimuleerde t-PA expressie in endotheelcellen sterk onderdrukte.

De inductie van t-PA door RA in gekweekte humane endotheelcellen verloopt langzaam. Dit was de reden om te onderzoeken of RARα de t-PA expressie direct of indirect induceert (hoofdstuk 3). In aanwezigheid van de eiwitsynthese remmer cycloheximide werd de inductie van t-PA door RA volledig geremd. Deze bevinding was een aanwijzing dat RARα de t-PA synthese niet direct stimuleerde, maar indirect via een ander eiwit. Aangezien de inductie van RARβ door RA precies vooraf ging aan de inductie van t-PA door RA, hebben we onderzocht of dit RAR subtype de uiteindelijke mediator van t-PA inductie is. Het bleek dat RA in gekweekte humane endotheelcellen met verhoogde RARβ mRNA niveau's (ten gevolge van een voorbehandeling van de cellen met RA en/of cycloheximide) de synthese van t-PA stimuleerde zonder vertraging. De hypothese dat RARβ de uiteindelijke mediator van de t-PA inductie is, werd versterkt door het uitvoeren van antisense experimenten. Een antisense oligodeoxynucleotide gericht tegen de translatie start plaats van RARβ2 mRNA onderdrukte de inductie van t-PA door RA. Een antisense oligodeoxynucleotide die RARβ4 synthese blokkeerde was niet effectief. De resultaten uit deze studies suggereren een 2-staps mechanisme voor de inductie van t-PA door RA, waarbij eerst RARβ2 wordt geïnduceerd door RARα en vervolgens RARβ2 de expressie van t-PA induceert.

Aangezien *in vitro*, in gekweekte humane endotheelcellen, hoge RA concentraties nodig zijn om de t-PA synthese te stimuleren vergeleken met de *in vivo* situatie, is het metabolisme van all-*trans* RA en 9-*cis* RA (een isomeer van all-*trans* RA) bestudeerd in gekweekte humane endotheelcellen. Ter vergelijking zijn dezelfde studies ook uitgevoerd in de humane hepatoma cellijn HepG2 en primaire kweken van humane hepatocyten, een erkend celtype voor RA metabolisme (hoofdstuk 4). Onze resultaten tonen het volgende aan: (1) Humane endotheelcellen metaboliseren all-*trans* RA snel, met een vergelijkbare snelheid als humane hepatocyten. Bovendien induceert all-*trans* RA zijn eigen metabolisme in humane endotheelcellen, maar niet in humane hepatocyten; (2) In tegenstelling tot all-*trans* RA wordt 9-*cis* RA erg langzaam gemetabolizeerd door endotheel cellen. Hepatocyten daarentegen metaboliseren 9-*cis* RA zelfs sneller dan all-*trans* RA; (3) Hepatocyten zijn in staat om all-*trans* RA te isomeriseren tot 9-*cis* RA en vice versa, terwijl endotheelcellen dit niet kunnen; (4) De cytochroom P450 remmers liarozole en ketoconazole remmen het metabolisme van all-*trans* RA in beide celtypen: de mate waarin deze stoffen remmen is echter verschillend voor beide celtypen. In aanwezigheid van de meest potente RA metabolisme remmer in endotheelcellen, liarozole, is een tenminste 10 maal lagere concentratie RA nodig om dezelfde inductie van t-PA te veroorzaken. Deze resultaten laten zien dat een een snel metabolisme van all-*trans* RA door endotheelcellen kan verklaren, waarom relatief hoge RA concentraties nodig zijn om t-PA synthese *in vitro* in gekweekte humane endotheelcellen te stimuleren.

Verschillende studies hebben een negatieve correlatie aangetoond tussen plasma concentraties van oestrogenen en die van t-PA. In hoofdstuk 5 werd de hypothese getest dat oestrogenen de plasma niveau's van t-PA verlagen door de klaring van t-PA te stimuleren. Wij hebben aangetoond dat ethinyloestradiol behandeling de klaring van intraveneus toegediend (exogeen), humaan t-PA in muizen vergroot. De klaring van eigen (endogeen) t-PA in ratten is ook verhoogd na ethinyloestradiol behandeling. Twee verschillende receptoren zijn verantwoordelijk voor de klaring van t-PA; de mannose receptor en de α2-macroglobuline receptor (ook wel LDL-receptor related protein (LRP) genoemd). Onze resultaten suggereren dat een verhoging van het aantal mannose receptoren door oestradiol verantwoordelijk is voor de toegenomen t-PA klaring: In de eerste plaats bleef het verschil in t-PA klaring tussen controle en ethinyloestradiol behandelde muizen aanwezig tijdens blokkade van de LRP door een natuurlijke antagonist, RAP. Aan de andere kant was de klaring van t-PA identiek in controle- en ethinyloestradiol-behandelde muizen in de aanwezigheid van een mannose receptor antagonist, mannan. In de tweede plaats veroorzaakte ethinyloestradiol behandeling een 6-voudige inductie van lever mannose receptor mRNA niveau's, terwijl de hoeveelheid mRNA voor LRP niet veranderde en gelijk was aan die van controle dieren. Door respectievelijk Western blotting en ligand blotting werden deze resultaten op eiwit niveau bevestigd. Samengevat tonen deze studies dat ethinyloestradiol de plasma klaring van t-PA vergroot door een sterke inductie van de mannose receptor. Deze resultaten kunnen de negatieve relatie tussen oestrogeen status en plasma t-PA concentraties verklaren.

Het onderzoek beschreven in hoofstuk 6 is uitgevoerd om het effect van verschillende hormonen op het angiogenese proces te bestuderen. Als model systeem voor angiogenese is een *in vitro* model gebruikt waarin bovenop een fibrine matrix humane microvasculaire endotheelcellen werden gekweekt in de aanwezigheid van tumor necrosis factor α (TNFα) en basic fibroblast growth factor (bFGF). Testosteron en dexamethason remden de vorming van tubulaire structuren in dit model bijna volledig, terwijl RA de vorming hiervan in de aanwezigheid van TNFα en bFGF juist sterk vergrootte. Parallel hieraan veranderden deze componenten de u-PA mRNA en eiwit niveau's op een vergelijkbare manier: testosteron en dexamethason remden, en RA stimuleerde de u-PA expressie. Resultaten van studies waarin u-PA werd toegevoegd of waarin de u-PA activiteit werd geïnactiveerd door antilichamen, maken het erg aannemelijk dat deze hormoon- geïnduceerde veranderingen in u-PA niveau's verantwoordelijk zijn voor de waargenomen veranderingen in angiogene activiteit. Door receptor specifieke antagonisten te gebruiken werd aangetoond dat deze componenten waarschijnlijk via hun nucleaire receptoren werken. De afwezige of slechts kleine effecten van 17β-estradiol, progesteron, schildklier (thyroid) hormoon en 1,25-dihydroxyvitamine D3 op *in vitro* angiogenese kunnen, met uitzondering van thyroid hormoon, verklaard worden door de afwezigheid van een significante expressie van desbetreffende nucleaire receptoren.

In hoofdstuk 7 worden enkele specifieke resultaten uit dit proefschrift in een bredere context besproken.

ABBREVIATIONS

bFGF	basic fibroblast growth factor
CHX	cyclohexamide
DMEM	Dulbecco's modified Eagle's medium
ER	estrogen receptor
EE	ethinylestradiol
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hMVEC	human microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
kb	kilo basepair (s)
LRP	low-density-lipoprotein receptor-related protein
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
RA	retinoic acid
RAR	retinoic acid receptor
RAP	receptor associated protein
RXR	retinoid X receptor
TNFα	tumor necrosis factor-α
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator

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Curriculum Vitae

Mirian Lansink werd op 26 september 1970 geboren in Den Haag. In 1988 behaalde zij haar Gymnasium β diploma op het Christelijk Lyceum in Gouda. In september van dat jaar begon zij met de studie Biomedische Wetenschappen (toen nog Gezondheidswetenschappen) aan de Rijksuniversiteit Leiden en in september 1989 behaalde zij het propaedeutisch examen (cum laude). In de doctoraalfase deed zij 3 stages van 3 maanden bij de respectievelijke vakgroepen: Infectieziekten onder leiding van Dr. P. Nibbering, Klinische Epidemiologie en Reumatologie onder leiding van Dr. A de Boer en Dr. J.M. Hazes en Medische Microbiologie/Virologie onder leiding van Dr. W. Luytjes. Tijdens haar afstudeerstage bij TNO-IVVO te Leiden deed zij onderzoek aan de interactie tussen u-PA en de u-PA receptor onder leiding van Dr. P.H.A. Quax. De studie werd in april 1993 cum laude afgerond.
Van mei 1993 tot mei 1997 is zij werkzaam geweest als AIO op het Gaubius Laboratorium TNO Preventie en Gezondheid in Leiden. Binnen de sectie pathofysiologie van het endotheel werd onder leiding van Dr. T. Kooistra en Prof. Dr. P. Brakman onderzoek verricht zoals in dit proefschrift beschreven staat. Het onderzoek werd gesubsidieerd door de Nederlandse Hartstichting. Sinds oktober 1997 werkt zij als onderzoeker bij Numico Research in Wageningen.

Nawoord

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