Retinoids in the modulation of vascular inflammation

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Stockholm 2005
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To my lovely wife Ewa and my wonderful daughters Amanda, Tilda & Nora
Abstract

Vascular disease is multifactorial. Smooth muscle cells, the major constituent of the normal vessel wall, play a pivotal role. The pathogenesis includes cellular differentiation, proliferation and inflammatory activation. Retinoids have been shown to influence all these processes and have therefore been identified as potential therapeutic agents in vascular pathology. However, knowledge about the role of retinoids in vascular disease is limited. The aim of this thesis was to investigate the effects of retinoids on vascular inflammation and vascular injury with special focus on vascular SMCs.

In manifest atherosclerosis with impaired blood flow due to reduced vessel diameter, therapeutic endovascular interventions including angioplasty and stent implantations are performed. The long-term outcome of these interventions is negatively influenced by the development of restenosis, in which proliferation of vascular SMCs is a key process. Retinoids are known regulators of cellular proliferation. We explored this mechanism and identified a retinoic acid receptor-\(\alpha\) mediated inhibition of SMC growth. We also showed that retinoids inhibit neointima formation after vascular angioplasty, resulting in increased luminal diameter.

Inflammation is a significant component of many forms of vascular pathology. In atherosclerosis, the inflammation is chronic, localized, low-grade and restricted to large arteries. In septic shock, the inflammation is acute, intense and generalized. Although clinically diverse, these processes share properties at the molecular level. Nitric Oxide (NO) is an important regulator of the homeostasis in the vessel wall and offer protection against an early phase of atherogenesis. However, high concentrations, produced by the inducible nitric oxide synthase (iNOS) in activated SMCs, are pro-inflammatory. The high local NO concentrations seen in atherosclerosis may cause cell- and tissue damage, whereas the high systemic levels in septic shock may contribute to vasoplegia and multiple organ failure. We hypothesized that retinoids exert some of their modulatory effects on inflammation through the iNOS pathway. Our results showed that all-trans retinoic acid, the biologically active retinoid ligand, inhibits iNOS transcription and thereby NO production in cytokine-stimulated vascular SMCs through the nuclear Retinoic Acid Receptor-\(\alpha\). In addition, we showed increased survival in endotoxemic rats when treated with synthetic retinoid agonists.

Retinoid receptors act as ligand-activated transcription factors, which require active retinoid ligands intracellularly. Ligands may originate from intracellular synthesis or uptake of preformed active retinoid ligands from extracellular sources. Vascular SMCs are naturally exposed to the plasma content of circulating retinoids. Since the plasma concentration ratio of all-trans RA to all-trans ROH is about 1:1000, the availability of the active ligand is very limited compared to the inactive pre-form. Thus, cellular biosynthesis of all-trans RA may strongly influence intracellular concentrations of active retinoid ligands and hence the transcriptional activity. Nonetheless, the role of endogenous retinoid ligands in the regulation of genes of vascular importance has not, so far, retained much interest. The modulatory effect of retinoids on pathological processes in the vascular wall has almost exclusively been studied in models of exogenous administrated active retinoid ligands. We therefore aimed to investigate the metabolism of retinoids and the generation of active retinoid ligands in vascular SMCs. These cells were shown to express several metabolizing enzymes and were competent producers of active retinoid ligands. Interestingly, we found that pro-inflammatory cytokines increase the production of active retinoid ligands in vascular SMCs. Hence, a link between endogenous retinoid metabolism and vascular inflammation was identified.

Realizing the potential importance of retinoid metabolism for the regulation of vascular inflammation, we compared the ability of biosynthesis of active retinoid ligands between intimal and medial vascular SMCs. Since intimal and medial SMCs are phenotypically different, we hypothesized that they differ in their capacity to synthesize active retinoid ligands. Intimal SMCs displayed increased retinoid metabolism and subsequent increased production of active retinoid ligands compared to medial SMCs. Thus, retinoid metabolism is linked to the phenotype of SMCs.

In summary, our studies suggest that the availability of active retinoid ligands in vascular smooth muscle cells influences the vascular response to inflammation and damage. The recognition of retinoids as important modulators in prevalent vascular pathology implies a potential therapeutic role for these agents in the treatment of certain vascular diseases.

Key words: retinoids, metabolism, vascular smooth muscle cell, nitric oxide, inflammation, proliferation, restenosis
List of Publications

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<tbody>
<tr>
<td>All-trans RA</td>
<td>all-trans retinoic acid</td>
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<tr>
<td>All-trans ROH</td>
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<tr>
<td>9-cis RA</td>
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<td>activating protein-1</td>
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<td>basic fibroblast growth factor</td>
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<tr>
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<tr>
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<td>vascular cell adhesion molecule-1</td>
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<td>vascular endothelial growth factor</td>
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Introduction

In a traditional view, vascular smooth muscle cells (SMCs) were considered as differentiated, quiescent cells dedicated to vasomotor function. However, SMCs are now considered to display multiple functions including regulation of extracellular matrix (ECM) composition as well as producers and targets for growth factors and pro-inflammatory cytokines, all important factors in the development of vascular diseases such as atherosclerosis and restenosis 1.

Retinoids, natural and synthetic derivatives of vitamin A, exert broad biological effects and are used clinically to treat a variety of dermatological diseases such as psoriasis and acne vulgaris as well as neoplastic diseases such as promyelocytic leukemia, all associated with processes such as dedifferentiation, hyperproliferation and inflammation. A growing number of studies have reported modulating effects of retinoids on processes such as cell migration, proliferation, matrix remodeling, coagulation and inflammation, all of which impinge on vascular diseases.

This thesis is focused on retinoids and vascular smooth muscle cells in the context of vascular inflammation and injury.

1 Vascular inflammation

Inflammation is an indispensable protective response by the body's system of self-defense. Inflammation is recognized as part of the non-specific (innate) immune response and is represented by calor, dolor, rubor and tumor, the four classical cardinal signs of inflammation. The innate immune system is limited to the recognition of evolutionarily highly conserved pathogen motifs and is considered as a first line of defense. Rapidly mobilized arms of innate immunity include phagocytotic leukocytes, complement, and inflammatory mediators. In addition, adaptive immunity, with its T and B cells, antibodies, and immunoregulatory cytokines, powerfully modulate the inflammatory process. The adaptive immune response includes the release of pro-inflammatory mediators which further promote the inflammation. The inflammatory reaction directs components of the immune system to the site of injury or infection. It is manifested by increased blood supply and vascular permeability, which allows neutrophils and mononuclear cells to leave the intravascular compartment, attach and migrate through an activated endothelium.
along a chemotactic gradient into the vessel wall. Localized host defense reactions and pathways of immuno-pathological tissue damage often involve substantial alterations in the function of blood vessels. Endothelial cells, strategically located at the interface between blood and tissues, regulate important processes in the inflammatory reaction such as expression of adhesion molecules and chemokines\(^2\). Furthermore, vascular SMCs, when activated, produce adhesion molecules, pro-inflammatory cytokines and chemokines and participate in ECM turnover\(^3\), thus play a prominent role in the vascular response to an inflammatory challenge.

Vascular inflammation is the culprit process in the pathogenesis of many diseases such as atherosclerosis and septic shock. In atherosclerosis, the inflammation is chronic, localized, low-grade and restricted to large arteries. In septic shock, the inflammation is acute, intense and generalized. Although clinically diverse, these processes share properties at the molecular level. One potentially important pathogenic factor is circulating endotoxin. In low concentrations, it may aggravate the chronic inflammatory disease of atherosclerosis\(^4\) and at high concentrations initiate a life-threatening state of sepsis and septic shock\(^5\). In both cases, the same basic cellular signaling pathways of the innate immune system are activated. Thus, common vascular inflammatory mechanisms play a decisive role in the development of prevalent diseases and a thorough understanding of the cellular signal transduction is of importance for the development of causal interventions.

### 1.1 Signaling in vascular inflammation

To elicit a cellular response to inflammatory stimuli, intracellular signal transduction pathways are activated which subsequently lead to gene transcription. Lipopolysaccharides (LPS), a glycolipid that comprises most of the outer leaflet of the outer wall of Gram-negative bacteria, activate endothelial cells lining the vascular wall as well as SMCs. LPS binds to the protein CD14, and the complex interacts with a family of pattern recognition receptors, called Toll-like receptors (TLRs). Upon ligand binding, these transmembrane receptors activate a downstream signaling cascade consisting of the scaffold protein MyD88, the IL-1 receptor associated kinase (IRAK) and tumor necrosis factor receptor-associated factor-6 (TRAF-6), which results in the activation of the transcription factor nuclear factor kappa-B (NFκB). NFκB is one of the most important transcription factors involved in the inflammatory response. It is retained in an inactive state in the cytoplasm through the masking of
their nuclear localization sites the inhibitory protein IκB. Degradation of IκB mediated by inhibitory kappa kinases (IKKs) liberates NFκB, which translocates to the nucleus where it binds to sequences in the promoter region of various inflammatory genes \(^6\). The nuclear import of NFκB allows transcriptional activation of over 100 genes that encode mediators of inflammation and immune responses. NFκB activation results in induction of adhesion molecules such as VCAM-1, cytokines such as IL-1, IL-6 and TNFα, chemokines such as MCP-1, procoagulant factors such as tissue factor as well as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 and endothelin-1. The NFκB signaling pathway is also activated by pro-inflammatory cytokines such as IL-1β and TNFα, which thereby further potentiates the inflammatory response through a positive feed-back loop. Taken together, this signal transduction pathway initiates an inflammatory response to the recognized host pathogen.

In parallel to the NFκB pathway, the vascular response to an inflammatory stimulus, and hence expression of inflammatory genes, is also mediated by another transcriptional activating pathway, the activating protein-1 (AP-1). AP-1 is composed of homo/heterodimers of proto-oncogenes c-fos and c-jun. Receptors with intrinsic tyrosine activity such as the platelet-derived growth factor (PDGF) receptor trigger, when activated, a phosphorylation cascade that leads to activation of the mitogen-activated protein kinase (MAPK). The activation of MAPK induces the expression of c-fos and leads to formation of a fos/jun complex called AP-1 and enhances the binding of AP-1 to sequences located in the promoter region in many inflammatory genes such as the iNOS. AP-1 is also important in the proliferation of vascular cells seen in response to vascular inflammation/injury \(^7,8\).
Figure 1. Summary of signal transduction pathways and gene products in activated SMCs. Nuclear Factor-kappa B (NFκB) and Activating Protein-1 (AP-1) translocate into the nucleus and bind to their corresponding binding sites within the promoter region, thereby activating gene transcription.

In summary, initiators of vascular inflammation such as LPS from invading microorganisms and pro-inflammatory cytokines activates several signal transduction pathways through a sequence of steps, which allows transcription factors such as NFκB and AP-1 to activate the transcription of many genes involved in the inflammatory response of the vascular wall. One of the key mediators of the response to vascular insults, the iNOS, is regulated in this way, that is, through the NFκB and AP-1 systems.
1.2 Nitric oxide and vascular SMCs

Nitric oxide (NO) was originally discovered as a modulator of the vascular tone \(^9,10\) but has later been found also to be a mediator of the innate immune defense to host pathogens. NO participates in the inflammatory process within the vascular wall by exerting multiple functions such as modulation of genes encoding adhesion molecules, cytokines and chemokines \(^11\). NO is produced by members of the nitric oxide synthase (NOS) family. These enzymes mediate the oxidation of L-arginine and molecular oxygen utilizing NADPH as an electron donor and using heme, FMN, FAD and tetrahydrobipterin as cofactors.

NO activates cytoplasmic soluble guanylate cyclases which generate cGMP \(^12,13\). cGMP is a key regulator of vascular SMC contractility, growth and differentiation, and regulates gene activity both positively and negatively at transcriptional as well as posttranscriptional levels \(^14\).

![Figure 2. Synthesis of Nitric Oxide](image)

Constitutive production of NO by endothelial NOS (eNOS, also called NOS1) is tightly regulated by intracellular calcium levels and is generally believed to account for the homeostasis of the vessels i.e. regulate vascular tone, inhibit platelet adhesion, leukocyte chemotaxis and inhibit SMC migration and growth \(^15\). A crucial mechanism underlying the anti-inflammatory actions of NO is based on inhibition of NF\(\kappa\)B activation \(^16\), suggesting tonic inhibition of NF\(\kappa\)B under basal conditions. Reduction in the activity of vascular NO, as manifested by an impaired endothelium-dependent vasodilation, is seen in both early as well as in manifested states of atherosclerosis \(^17\). Failure of NO release from the endothelium with normal physiological stimuli provides conditions propitious for leukocyte adhesion, vasospasm and thrombosis and, in addition, may promote increased proliferation of intimal SMCs. Indeed, when atherosclerosis prone apolipoprotein E deficient mice were mated with eNOS
deficient mice, increased atherosclerosis was seen associated with hypertension, supporting protective effects of eNOS-derived NO in the pathogenesis of atherosclerosis.

Figure 3. Nitric oxide regulates homeostasis within the vascular wall. Modified picture from Barbato et al J Vasc Surg 2004.

In contrast to eNOS, the NO synthase involved in the innate immune system is expressed after transcriptional activation of the inducible NOS isoform (iNOS, also called NOS2) by pro-inflammatory cytokines or LPS. This leads to sustained production of large amounts of NO since iNOS is not dependent of intracellular calcium levels. The iNOS promoter contains several binding sites for transcription factors such as NFκB and AP-1. Cytokine activation of vascular SMCs induces the iNOS pathway that produce large amount of NO in the vessel wall, which may inhibit thrombi formation and vasospasm at sites of injury. Furthermore, NO synthesized via the iNOS pathway in activated macrophages is necessary for the killing of bacteria and inhibits viral replication. Although protective in the normal defense of the vessel wall, excessive amounts of iNOS-derived NO can be toxic and pro-inflammatory. In contrast to basal NO produced by eNOS, high concentrations of iNOS-derived NO have been shown to induce NFκB activity in mice suffering from hemorrhagic shock. Furthermore, high local concentration of NO can induce nitrosylation of enzymes in the mitochondrial electron transport chain, leading to inhibited mitochondrial respiration. It may also induce apoptosis in SMCs as well.
as tumor cells. This may be the scenario in chronic inflammatory diseases such as atherosclerosis. High levels of iNOS expression have been detected in macrophages and SMCs in human atherosclerotic lesions. Also, high concentration of iNOS-derived NO may react with oxygen species to form cytotoxic peroxynitrite radicals, potentially causing injury to the endothelium and SMCs, including apoptosis, and this may be a factor in atherosclerosis leading to plaque weakening. This hypothesis is further supported by the finding that atherosclerotic plaque formation is reduced in apoE-iNOS double knock out mice compared to the apoE mice. However, the effect of iNOS on intimal hyperplasia after vascular interventions is at present not fully elucidated, since both a decrease as well as an increase of intimal hyperplasia has been reported in iNOS-deficient mice. Taken together, the data above indicate that basal NO produced by eNOS exercises tonic inhibition of the inflammatory response, whereas high iNOS-derived NO may promote inflammation. Thus, inflammation related diseases such as atherosclerosis may be attenuated by low levels of NO, and augmented by high NO levels.

Figure 4. Pro-inflammatory and cytotoxic effects of nitric oxide on the vascular wall.

In contrast to the high local concentrations of NO seen in atherosclerotic lesions, a general induction of iNOS and high systemic concentration of NO is seen in patients suffering from sepsis and septic shock. Several studies have shown a significant rise in plasma levels of nitrate and nitrite, stable bioreaction products of NO, in patients
with septic shock\textsuperscript{34,35}. Furthermore, studies with mice lacking the iNOS gene show resistance to LPS-induced septic shock\textsuperscript{36}. Thus, excess production of NO appears to be linked to the hypotensive and vasoplegic state leading to tissue damage and organ dysfunction characteristic for septic shock\textsuperscript{37}. Since high levels of NO may account for many of the deleterious effects seen in sepsis, many studies have tried NOS/iNOS-blockers for treatment of various models of septic shock. Although many pathological processes in septic shock, including vasoplegia, are believed to be mediated through the iNOS/NO pathway, iNOS-independent routes do exist\textsuperscript{38}. Thus, contradictory results are seen with a short transient effect as well as no effects or even harmful effects, which address the complexity of the nitric oxide pathway in the state of septic shock\textsuperscript{39}. This could be due to the fact that most NOS inhibitors not only inhibit the iNOS-pathway but also decrease the production of the protective eNOS-derived NO. This could lead to impaired organ perfusion and hence may enhance the multiple organ failure often seen in patients suffering from septic shock.

2 Vascular injury – role of SMCs

Inhibitory actions that maintain quiescence of SMCs derive both from cell-matrix interactions and soluble mediators. However, at sites of vascular injury such as after endovascular procedures, a complex web of cellular and molecular responses, including the interaction of platelets, leukocytes, and the coagulation-fibrinolysis cascades, as well as the secretion of growth factors and pro-inflammatory cytokines, contributes to neointimal hyperplasia and the development of restenosis. These processes ultimately result in dedifferentiation, migration and growth of SMCs, which subsequently lead to intimal hyperplasia and restenosis\textsuperscript{1}. Restenosis is defined as a reduction in lumen size of an atherosclerotic artery after an intra-arterial intervention such as balloon angioplasty and stenting\textsuperscript{40}. It is unfortunate that all forms of vascular reconstruction inevitably causes some damage to vessels, and the healing often causes narrowing of the lumina. It limits the outcome of endovascular interventions and represents a clinical problem.

2.1 Molecular mechanisms in intimal hyperplasia

The vascular response to injury can be schematically divided into four phases: (1) a mechanical phase, (2) a thrombotic phase, (3) a proliferative phase in which
proliferating SMCs form a neointima, and (4) a remodeling phase with extracellular matrix deposition and reendothelialiation.

Endothelial denudation exposes ECM constituents, like collagen in the subendothelial thrombogenic layer, which results in platelet adhesion and thrombus formation. Platelets release various cytokines and growth factors, including IL-1, IL-8, transforming growth factor beta (TGFβ), platelet-derived growth factor (PDGF) and thrombin. Thrombin acts both as a mitogen for SMCs as well as a chemoattractant for leukocytes. Thrombin also induces expression of tissue factor on cultured SMCs. Intimal SMC expression of tissue factor plays an important role in the thrombus formation and subsequent neointimal development following balloon-injury and inhibition of tissue factor reduces intima development. The thrombotic cascade plays a prominent role in the initial recruitment of inflammatory cells. Vascular cells such as SMCs produce, when activated, growth factors, chemokines and cytokines which further initiate and potentiate the inflammatory response.

The proliferative phase includes phenotypic modulation of SMCs (discussed in more detail in the section below), migration towards the intima and SMC growth, resulting in the formation of a neointima. In order to migrate, SMCs need ECM remodeling, cytoskeletal rearrangement and a chemo-attractant gradient towards the intima. The ECM components can regulate the activated state of SMCs and may exert a negative growth control of these cells. In addition, ECM interactions may also control the availability and activity of growth factors, which is supported by the finding that basic fibroblast growth factor (bFGF) does not stimulate SMC proliferation in normal, uninjured vessels. Downregulation of β1-integrin, which anchor SMCs to ECM constituents, correlates with increased proliferative and migratory activities of SMCs. Matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of MMPs (TIMPs) play an important role in ECM remodeling and MMP inhibitors have been shown to reduce neointima formation. MMPs are produced by inflammatory cells (i.e. macrophages) as well as SMCs. Vascular SMCs have a basal production of pro-MMP-2 and produce other MMPs, such as MMP-9, upon cytokine stimulation, after vascular intervention as well as in atherosclerosis. Furthermore, upregulation of plasminogen activators (urokinase plasminogen activator, uPA, and tissue plasminogen activator, tPA) leads to plasmin
activation, which activates latent intracellular MMPs. Thus, these processes reduce
the integrity of the vessel which is a prerequisite for SMC proliferation. However,
these processes are balanced by factors that mediate ECM production such as TGFβ,
which stimulate collagen synthesis \(^{51}\), inhibit SMC proliferation \(^{52,53}\) and decrease
migration \(^{54}\). Furthermore, TGFβ contributes to enhanced expression of TIMPs in
SMCs \(^{55}\) and has potent anti-inflammatory action in the vessel wall \(^{56}\). SMCs have
been shown to express several Smad proteins, responsible for the intracellular
signaling of TGFβ \(^{57}\).

PDGF, produced and released by platelets as well as SMCs within the intima, act as a
chemoattractant encouraging phenotypically modified SMCs to migrate from the
media into the neointima where they continue to proliferate. Furthermore, PDGF
induces a rapid downregulation of differentiation markers in cultured SMCs \(^{58}\) which,
as mentioned, precede SMC migration. Tissue factor, comparable with PDGF, in
complex with factor VIIa induces chemotactic migration of cultured rabbit SMCs \(^{59}\).
Hence, activated, proliferating SMCs migrate through a modulated ECM into the
intimal space where they continue to proliferate when exposed to various growth
factors.

The first wave of proliferation is mediated by bFGF from disrupted cells \(^{60}\) and is
further augmented by the generation of thrombin \(^{61}\). Other factors that may play a
significant role in intimal hyperplasia include angiotensin II \(^{62}\) and endothelin \(^{63}\).
After migration into the intimal space, a second wave of proliferation takes place.
Activated platelets, leukocytes, endothelial cells (ECs) as well as SMCs produce a
variety of growth factors, such as PDGF, bFGF, TGFβ, insulin-like growth factor-1
(IGF-1) and vascular endothelial growth factor (VEGF), which all are mitogenic
factors to SMCs. Furthermore, the mitogenic response to growth factors is increased
in intimal SMCs compared to medial cells, probably due to increased expression of
IGF-1 receptor and PDGF-receptor beta \(^{64}\). The production of growth factors by
stimulated SMCs implies a potential positive feed-back loop with sustained SMC
growth, which may explain how the process of neointima formation continues after
the disappearance of the initial stimuli.
In contrast, immuno-competent cells produce the cytokine such as IFN\(\gamma\), which inhibits SMC proliferation and reduces ECM synthesis\(^{51,65}\). Noteworthy, vascular SMCs have been shown to produce IL-1\(^{66}\), TNF\(\alpha\)\(^{67}\), IL-6\(^{68}\) as well as IFN\(\gamma\)\(^{69}\) when activated, which can act in a paracrine fashion. IFN\(\gamma\) is a potent growth-inhibitory cytokine on SMCs through blocking the G1-phase in the mitogenesis. It also reduces ECM production and hence inhibits intimal hyperplasia\(^{70,71}\). Thus, the effects of IFN\(\gamma\) on SMCs may explain why inflammation destabilizes atherosclerotic plaques\(^{72}\). In conclusion, the growth stimulatory mediators that promotes SMC proliferation and intimal hyperplasia is balanced by the presence of pro-inflammatory cytokines that inhibit SMC proliferation and reduce vascular integrity.

**Figure 5.** Histology of the rat vessel wall. Normal anatomy (A) and intimal hyperplasia after endo-vascular intervention (B). Both sections stained for CRBP (black).
2.2 Heterogeneity of vascular SMCs

The normal differentiated medial SMCs exhibit very low rates of proliferation \(^{73,74}\). Nevertheless, SMCs retain the ability to migrate and divide rapidly in response to injury \(^{75}\). Such a change in behavior naturally requires a switch in the spectrum of active genes, generally referred to as “phenotypic modulation”, which is a prerequisite for migration and proliferation \(^{76}\). The paradigm during the years has been that the combined action of growth factors, proteolytic agents and extracellular matrix components, produced by a dysfunctional endothelium and/or inflammatory cells induces phenotypic modulation of SMCs with proliferation and migration towards the intima. Alternative to this hypothesis that all SMCs of the media can undergo phenotypic modulation is the concept that a predisposed SMC subpopulation is responsible for the production of the intimal thickening. It has been shown by several groups that SMC heterogeneity exists within the vessel wall varying from the adult rat to humans \(^{77,78}\). SMC heterogeneity can be morphologically divided into two phenotypes, epithelioid and spindle-shaped, which coincide with the functional classification of synthetic and contractile phenotypes, respectively \(^{79,80}\). In culture, medial SMCs have a spindle-shaped morphology and exhibit the classic “hill-and–valley” growth pattern, whereas intimal SMCs show an epitheloid phenotype, grow as a monolayer and exhibit cobblestone morphology at confluence. Once seeded, the two SMC phenotypes maintain their distinct features \(^{81}\), indicating that the phenotype of SMCs depends more on their intrinsic features rather than their environment, thereby reinforcing the notion of SMC heterogeneity. The epitheloid phenotype is sometime referred to as a non-muscle phenotype since it lacks the contractile differentiation markers and does not respond with contraction to increased intracellular calcium concentration.

The two phenotypes demonstrate different gene expression patterns \(^{82,83}\), where re-expression of fetal genes are generally seen in the synthetic phenotype \(^{84}\). Intimal SMCs have been shown to overexpress or even uniquely express certain genes including those encoding cytokines, adhesion molecules, growth factors and ECM proteins. So far, only three genes are considered to be “intima-specific”, cytokeratin 8 and 18 \(^{85}\) and cellular retinol binding protein-1 (CRBP-1) \(^{86,87}\). Interestingly, CRBP-1 (discussed in more detail in section 3.2.1) is involved in retinoid metabolism, indicating altered metabolism of these molecules in this subset of SMCs. After endothelial injury, CRBP-1 is expressed in a subset of medial SMCs and in the vast
majority of SMCs in the intimal thickening, which further supports the hypothesis that a subset of predisposed medial SMCs is responsible for neointima formation.

Intimal SMCs have also been proposed to originate from diverse sources, including fibroblasts of the adventitia, endothelial cells and/or circulating bone marrow-derived progenitor cells. The importance of each source of intimal SMCs in the generation of intimal hyperplasia is much under debate and the contribution to intimal hyperplasia seems to depend on injury model.

Figure 6. Intimal (A) and medial (B) rat smooth muscle cells in culture.

3 Retinoid metabolism and signaling

Retinoids, i.e. vitamin A and its active metabolites, modulate many processes implicated in the pathogenesis of vascular inflammation and injury. Retinoids exert their cellular effects on the transcriptional level. For this to occur, active retinoid ligands are needed. Retinoid metabolism and generation of active retinoid ligands is complex involving multiple binding proteins and metabolic enzymes and has not in detail been fully investigated. To obtain transcriptional activity, ligand-activated retinoid receptors interact with the complex web of the cellular transcriptional machinery. Hence, for the sake of simplicity, the summary below does not detail all possible metabolic pathways and known control points, but rather focuses on selected events in the generation of retinoid ligands and how they regulate gene transcription.

3.1 Retinoid transport and uptake into target cells

Retinol (ROH), the substrate in the biosynthesis of active retinoid ligands, is mainly stored in the liver. Due to their hydrophobic nature, retinoids need association with
retinoid-binding proteins in aqueous environments. ROH is transported to the target cells bound to retinol-binding protein (RBP) at a concentration between 2-3 µM. In addition, low levels of pre-formed, biologically active, retinoic acid (RA) circulate in plasma (5-10 nM), presumably bound to albumin. The mechanism of uptake of retinol into target cells has not been conclusively established and both a receptor-independent mechanism, in which retinol dissociates from RBP and diffuses across the phospholipid membrane of a target cell, and a receptor-mediated uptake has been suggested. RBP knock-out mice have decreased levels of circulating retinol in plasma and increased hepatic retinol content. However, they are phenotypically normal, except for a visual impairment, which is corrected when they are put on vitamin A-sufficient diet, indicating that RBP neither is essential for transport of ROH to target tissues, nor for the uptake into target cells.

### 3.2 Intracellular retinoid binding proteins

It has become increasingly clear that the traditional view that intracellular retinoid-binding proteins act mainly as aqueous storage compartments for retinoids is far from complete. Rather, accumulated evidence indicates that many of these proteins play specific roles in regulating the transport, metabolism and action of their ligands.

#### 3.2.1 Cellular retinol-binding protein (CRBP)

In order to be protected from non-specific oxidation once inside the cell, retinol is associated with a cellular retinol binding protein. Three isoforms, CRBP-I, CRBP-II and CRBP-III, have been cloned. The three isoforms of CRBP display a strikingly different tissue distribution. CRBP-I is expressed in multiple tissues whereas CRBP-II expression is restricted to the small intestine, suggesting that CRBP-II is involved in the processing of dietary retinoids. Recently, CRBP-III was cloned in mouse and human and is expressed at highest level in the human kidney and liver. Interestingly, a recent study showed expression of CRBP-III in vascular endothelial cells. CRBP-I binds retinol with high affinity whereas CRBP-II binds both retinol and retinal with same affinity, however with 100 times lower affinity compared to CRBP-I.

CRBP is proposed to serve two main functions. It can direct retinol to storage, in presence of the enzyme lecithin:retinol acyltransferase (LRAT) that converts retinol to retinylesters (RE), or direct retinol into the metabolic generation of RA. LRAT is
inhibited by apo-CRBP (without ROH) and hence, decreased generation of retinylesters is seen in cells with low ROH content. Furthermore, apo-CRBP stimulates retinylester hydrolase (REH), which converts retinylesters into ROH, thus contributing to RE mobilization during times of retinol insufficiency. Overall, current information implicates CRBP-1 in the regulation of vitamin A storage, as well as the activity of the metabolic enzymes that produce biologically active RA. It is worth noting, however, that while the proposed function for CRBP in the regulation of vitamin A storage has been strongly supported by studies of CRBP-I-null mice, these animals do not display congenital abnormalities related to retinoic acid deficiency, at least under conditions of maternal vitamin A sufficiency. Hence, under these conditions, CRBP-I does not appear to be indispensable for RA synthesis during embryogenesis. Interestingly, intimal SMCs express high levels of CRBP-1, which appear stable in cell culture, compared to medial SMCs, indicating altered retinoid metabolism in this subset of cells. CRBP-1 upregulation is a direct transcriptional effect of RA, through binding of the RARα-RXRα heterodimer to the retinoic acid response element (RARE) of the CRBP-I promoter. Hence, a positive correlation exists between the level of CRBP-1 and retinoid responsiveness, and cells that metabolize retinoic acid are generally growth inhibited by all-trans RA.

3.2.2 Cellular retinoic acid-binding protein (CRABP)

RA, the biologically active retinoid ligand, needs, in parallel with ROH, protection against non-specific cellular oxidation. This is carried out by CRABPs, members of the cellular retinoid binding protein family, which bind RA with high affinity and discriminate against ROH. The two isoforms of CRABP display different patterns of expression across cells and stages of development. In the adult, CRABP-I is expressed almost ubiquitously, while CRABP-II exhibits a more restricted expression pattern such as the skin, uterus and ovary. Both CRABPs are expressed in the embryo, although they do not usually co-exist in the same cells. The biological function of CRABP is at present not fully understood. However, as in the case of CRBP, the reported knock-out mice lacking CRABP-I or CRABP-II had normal embryological development with no specific phenotype. Even the CRABP-I/CRABP-II double knock-out mice were normal, except for a minor defect in limb development. However, CRABP have been shown to be responsible for the nuclear translocation of all-trans RA. Furthermore, CRABP-I has been shown to
modulate the activities of enzymes that catalyze the metabolic transformation of retinoic acid, giving rise to polar metabolites.

These polar metabolites are products from Cyp 26, members of the cytochrome P450 family, which specifically metabolizes retinoic acid into polar metabolites, i.e. 4-oxy RA and 18-OH-RA, for cellular excretion. So far, three members of the P450RAI (RA-inducible) family have been cloned, Cyp26A1, Cyp 26B1 and Cyp 26C1. Cyp 26 is induced by all-trans RA through RA-induced transactivation of a RARE located in the Cyp 26 promoter, suggesting a role for Cyp 26 as a damper of the cellular response to retinoic acid. Indeed, Cyp 26A1 knock-out mouse fetuses have been shown to have lethal morphogenetic phenotypes mimicking those generated by excess retinoic acid administration. Thus, all-trans RA both acts as the substrate for and inducer of Cyp 26. Although these polar metabolites are considered as inactive, catabolic products, they may have biological effects since some bind to retinoid receptors. However, at present, their biological function remains unclear.

Figure 7. Chemical structures of some endogenous retinoids.

3.3 Retinoic Acid synthesis

The synthesis of the biologically active retinoids is a two-step reaction involving substrate-specific enzymes in the presence of cellular retinol binding proteins.
3.3.1 Oxidation of retinol by retinol dehydrogenases (RDHs)

The first step of the metabolic synthesis of retinoic acid is the reversible oxidation of retinol into retinal. This oxidation is mediated by enzymes belonging to the short-chain dehydrogenases/reductases (SDRs)\textsuperscript{122}. So far, at least ten cDNA clones have been isolated which encode microsomal members of the SDR family that catalyze the conversion of all\textit{-trans} retinol or various \textit{cis}-retinol isomers into the corresponding retinals. Each member shows a unique pattern of catalysis with various retinoids. Vascular SMCs express two members of SDRs, retinoldehydrogenase-2 (RDH-2) and retinoldehydrogenase-5 (RDH-5), which catalyze all\textit{-trans} retinol and 9-\textit{cis} retinol respectively (Gidlöf Biochem Biophys Res Commun 2001, paper 3 in this thesis).

Retinol can also be oxidized by a family of medium-chain alcohol dehydrogenases (ADHs). ADHs have been implicated in the general detoxification reactions of alcohols and aldehydes. The ADH type I and type IV metabolize free retinol (retinol that is not bound to CRBP) into retinal \textit{in vitro}\textsuperscript{123}. However, their physiological importance \textit{in vivo} remains controversial.

3.3.2 Oxidation of retinal by retinaldehydrogenases (RalDHs)

The second step, the oxidation of retinal into the biologically active retinoic acid, is irreversible and is carried out by aldehyde dehydrogenases. Several retinal dehydrogenases have been cloned. The two RalDHs that have been studied most are RalDH-1 and RalDH-2 that use both all\textit{-trans} retinal and 9-\textit{cis} retinal as substrate and metabolize it into the corresponding retinoic acid. The produced isoform of retinoic acid is however suggested to be able to isomerize into other isoforms. Whether this is an enzymatically process is still unknown. Vascular SMCs have been shown to express RalDH-1, whereas RalDH-2 was not detectable (Gidlöf, unpublished observation, paper 5 in this thesis).
3.4 Retinoid function in target tissue cells

Retinoids exert their function mainly through regulating gene transcription. Retinoid signaling is transduced through the action of nuclear hormone receptors that either activate or suppress gene expression.

3.4.1 The nuclear receptor family

Nuclear receptors are ligand-activated transcription factors that specifically regulate the expression of target genes. More than 100 nuclear receptors are known to exist. A typical nuclear receptor, as depicted in Figure 9, consists of a variable NH2-terminal region (A/B), a conserved DNA-binding domain (DBD), a linker region (D), and a conserved region that contains the ligand binding domain (LBD)\textsuperscript{124}. The A/B domain shows promoter- and cell-specific activity, suggesting that it is likely to contribute to the specificity of action among nuclear receptor isoforms and that it could interact
with cell type specific factors. The DBD is the most conserved region and confers the ability to recognize specific target sequences. This domain contains two zink fingers for DNA binding of the nuclear receptor. The D domain serves as a hinge between the DBD and the LBD and allows rotation of the DBD. It also harbors nuclear localization signals. The LBD binds the nuclear receptor ligand. It also mediates homo- and heterodimerization of the nuclear receptors and mediates ligand-dependent transcriptional activity.

**Figure 9.** Schematic picture of a typical nuclear receptor. Abbreviations: A/B: Variable domain; DBD: DNA Binding Domain (RARE); LBD: Ligand Binding Domain.

### 3.4.2 Retinoid receptors

Retinoid receptors encompass two nuclear receptor families, Retinoic Acid Receptors (RARs) and Retinoic X Receptors (RXR)\(^ {125-127} \). Each family consists of three isotypes (α, β and γ) encoded by separate genes. Furthermore, multiple isoforms of each isotype of the retinoid receptor is generated from a single gene by alternative splicing or alternative start sites of transcription. Retinoid receptors show a distinct expression pattern among tissues, where RARα and RARβ show the most general expression pattern whereas RARγ and RXRγ are expressed in a more restricted fashion\(^ {128,129} \). Vascular SMC have been shown to express all isotypes of retinoid receptors except RXRγ on the mRNA level\(^ {130} \). On the protein level, vascular SMCs express mainly RARα and RXRα\(^ {131} \). Indeed, most biological effects of retinoids on SMCs are through RARα.

RAR binds all-trans RA and 9-cis RA whereas RXR binds only 9-cis RA. After binding their ligand they form homo- or heterodimers in order to achieve transcriptional activities. RXR not only form a heterodimer with RAR, but also act as a permissive nuclear receptor that serves as heterodimerization partner to other nuclear receptors such as the peroxisome proliferator-activated receptor (PPAR). The RAR/RXR is a so-called non-permissive heterodimer that can be activated only by the RAR ligand but not by an RXR ligand alone\(^ {132,133} \). However, although a RXR
ligand alone cannot activate the heterodimer, binding of the RAR ligand allows the subsequent binding of the ligand of RXR, which then enhances the transcriptional response to the RAR ligand \(^{134}\). Knock-out studies of each retinoid receptor generated viable mice with modest phenotype except for RXR\(\alpha\), in which a severe cardiac abnormality and embryonic lethality was seen \(^{135,136}\). Despite the expression of five out of six retinoid receptors in SMCs, no vascular phenotype was detected in these mice. This may be due to a redundancy in retinoid receptor signaling. However, when combined retinoid receptor KO mice were created, severe and in some cases lethal phenotypes were seen, including major effects on the cardiovascular system \(^{137}\).

### 3.4.3 Retinoid signaling through Retinoic Acid Response Elements (RARE)

Retinoid receptors regulate gene transcription by binding to specific DNA sequences located in the promoter region in target genes known as Retinoic Acid Response Elements (RARE). RARE is composed of two hexameric motifs arranged as direct repeats (DR) separated by one to five base pairs (bp). The typical RARE for RARs is designed DR5 (two direct repeats separated by five bp). After binding to the response element they interact with several components of the transcriptional machinery and hence activate or repress gene transcription. Genes with a RARE in the promoter region is considered as early response genes for RA. In addition, the products of early response genes can activate the transcription of secondary genes. Transactivation of these genes therefore represents secondary action of retinoids since their transcription requires protein synthesis. The unligated retinoid receptor may have a nuclear localization with inhibitory effects on gene transcription through association with co-repressors responsible for the silencing activity. After ligand binding, the conformational changes in the receptors would cause the dissociation of co-repressors and recruitment of co-activator complexes responsible for transcriptional activation \(^{138}\). However, some nuclear receptors, such as the steroid receptors are cytoplasmic in the absence of ligand due to their association with a large multiprotein complex of chaperones, including Hsp90 and Hsp56 \(^{139}\).

In addition to the nuclear receptors themselves, coactivators and corepressors are required for efficient transcriptional regulation. The readers are referred to excellent review articles since it is out of the scope to handle in this thesis \(^{140,141}\).
3.4.4 Transcriptional antagonism and “cross-talk” with other signaling pathways

Nuclear receptors, including retinoid receptors, can also modulate gene expression by mechanisms independent of binding to their corresponding response elements. They can influence gene transcription in genes that do not contain a response element, through positive or negative interference with the activity of other transcription factors, a mechanism generally referred to as transcriptional cross-talk. One of the most studied examples is the negative interference with AP-1 (c-Jun/c-Fos) where RAR act as ligand-dependent transrepressor of the AP-1 activity. Many of the antiproliferative effects of nuclear receptors are believed to be due to cross-talk with AP-1. Some studies indicate a transcriptional cross-talk between retinoid receptors and other transcription factors such as NF-κB and NF-IL6. Noteworthy, these transcription factors play an important role in vascular inflammation and vascular injury. It was originally proposed that nuclear receptors such as the glucocorticoid receptor inhibit the DNA binding of AP-1 and NFκB to their corresponding cognate sites. However, more recent evidence suggests that a competition for common transcriptional cofactors could be involved in this antagonism. Additional mechanisms have been suggested, including an induction of the IκB that sequesters NFκB in the cytoplasm, or an inhibition of the Jun-NH2-terminal kinase (JNK) activity that would prevent phosphorylation of c-jun and, hence, AP-1 activity. However, the mechanism(s) for the transcriptional antagonism of retinoids in vascular SMCs remains to be further investigated.

4  **Review of retinoids in vascular biology/pathobiology**

Cardiovascular diseases such as atherosclerosis and restenosis involve polygenetic traits and multifactorial processes. Future possible treatments should have pleiotropic effects rather than pathway- or gene/protein specific activities. Retinoids are attractive candidates since they act as pluripotent modifiers on many processes involved in vascular diseases. Retinoids have been shown to regulate migration, proliferation, apoptosis, matrix remodeling, fibrinolysis/coagulation and inflammation, all of which impinge on vascular diseases.

![Figure 11. Summary of effects of retinoids on vascular processes.](image)

In this section I aim to review the effects of retinoids on important vascular processes with particular focus on SMCs. In the end of this section, a nonexhaustive list is presented with retinoid-regulated genes of vascular importance.

4.1  **Retinoids and matrix remodeling**

The vascular wall is composed of cells, where SMCs are the most abundant, embedded in extracellular matrix. Earlier seen as pure structural components, knowledge today include important functions of ECM in vascular homeostasis/integrity such as regulating migration of embedded cells and have been shown to harbour mitogenic and chemoattractive substances. Vascular cells regulate a
continuous balance between ECM synthesis and degradation. Retinoids have been shown to modulate both synthesis of ECM components as well as degrading enzymes in vascular SMCs both in vitro and in vivo. Retinoid treated SMCs showed decreased deposition of fibronectin, thrombospondin-1 and matrix Gla protein\textsuperscript{154,155} as well as increased expression of collagen-1 and elastin\textsuperscript{154,156}. Matrix degrading processes is mediated by matrix metalloproteinases (MMPs) as well as the plasminogen system, mainly tPA and uPA. Retinoid treated SMCs have been shown to downregulate at least 4 members of the MMP family (MMP1, MMP2, MMP3, MMP9)\textsuperscript{154,157-159}. Furthermore, these cells showed increased expression of TIMP-1, an endogenous MMP inhibitor\textsuperscript{157}. tPA is an activator of plasminogen from which plasmin is derived. Plasmin may induce the degradation of many extracellular proteins either directly or through the activation of latent MMPs\textsuperscript{160}. Intimal SMCs display increased expression of tPA and higher proteolytic activity compared to medial SMCs\textsuperscript{161}. Furthermore, all-trans RA increase the expression of tPA through a RARE within the promoter region\textsuperscript{162}. Hence, retinoids may increase the expression of ECM constituents, decrease matrix degradation through the MMPs and increase the proteolytic activity through the tPA system. However, retinoids also induce plasminogen activator inhibitor-1 (PAI-1) in vascular SMCs, thereby decreasing the proteolytic activity of the PA-system\textsuperscript{163}. Thus, the net effect of retinoids seems to favor ECM production with increased vascular integrity. However, the local activity, and balance, of proteolytic processes, i.e. MMPs and the PA-system, may relay on the experimental setup and influence the outcome of retinoid treatment. Decreased vascular integrity, seen in many vascular diseases such as atherosclerosis and restenosis, precedes many processes such as migration and cell proliferation in responses to vascular injury. Thus, retinoids may regulate these processes through modulation of the ECM composition and, hence, vascular integrity.

4.2 Retinoids and SMC migration

Migration of medial SMCs towards the intima is an early hallmark in the development of restenosis after endovascular interventions. Migration depends on ECM remodeling, cell-ECM adhesive properties as well as presence of chemotactic agents. Thus, the ECM composition and integrity play a major part in regulating cell migration within the vessel wall. Further, as mentioned, the ECM harbours chemotactic agents, which are released upon matrix degradation. In injured vessels,
β1-integrin, responsible for SMC-matrix adhesion, is downregulated \(^\text{164}\). Decreased expression of β1-integrin precedes phenotypic modulation of SMCs and allows the cells to migrate. Retinoids have been shown to upregulate β1-integrin \(^\text{165}\). This together with increased ECM and decreased matrix degradation through inhibition of MMPs should subsequently lead to decreased migration of SMCs. However, retinoids increase the expression of tPA, which is generally considered to be a facilitator of cell migration. Indeed, contradictory data do exist with both decreased migration rate \(^\text{154,159,166}\), as well as increased migration of retinoid treated SMCs \(^\text{167,168}\). These discrepant findings highlight the complexity of the migratory process of SMCs since retinoids have been shown to influence several processes that precede cell migration. The outcome seems to be related to the experimental setup and further studies are warranted to determine the net effect \textit{in vivo}.

### 4.3 Retinoids and SMC proliferation

In restenosis, vascular SMCs and ECM are the main constituents which ultimately reduce luminal size. Thus, SMC proliferation is believed to play a central role in this process. During the last years, contradictory reports on the proliferative effect of retinoids on vascular SMCs have emerged. An early finding showed mitogenic effects of all-\textit{trans} RA on SMCs \(^\text{169}\). Other reports showed no effect of retinoids on SMC proliferation \(^\text{170}\). However, a vast majority of the reports showed growth inhibitory effects of retinoids on vascular SMCs of different species from rats to human \(^\text{130,158,167,171}\). Overall, it seems that retinoids stimulate quiescent SMCs in contrast to mitogen-stimulated SMCs, in which growth inhibition is seen. Retinoids have been shown to inhibit the proliferative effect of several mitogenic factors on SMC such as PDGF-BB \(^\text{130,158}\), angiotensin II \(^\text{131}\), serum \(^\text{156}\), serotonin \(^\text{172}\), endothelin-1 \(^\text{173}\) and bFGF \(^\text{174}\). The mechanisms behind this inhibitory effect are still somewhat unclear and seem to involve multiple mechanisms in the mitogenic signaling pathway and are probably downstream of the early responses to mitogenic stimuli. This growth inhibition has been suggested to be located at cell cycle checkpoints since retinoids have been shown to target multiple genes for cyclins and cyclin-dependent kinases in SMCs \(^\text{171,173,174}\). Recently, the novel Krüppel-like zink-finger transcription factor 5 (KLF5) was identified and found to be markedly induced in activated vascular SMCs \(^\text{175}\). KLF5 is upregulated in the neointima within vascular lesions and the heterozygous KLF-KO (Klf5\(^{+/−}\)) mice show a marked decrease in intimal and medial thickening.
after vascular interventions compared to wild-type animals \textsuperscript{176}. Interestingly, the authors found that RAR-ligands affected KLF5 transcriptional activity by a direct physical interaction between KLF5 and ligand-activated RAR, thus indicating a putative mechanism in retinoid-mediated growth inhibition of activated vascular SMCs \textsuperscript{176}.

### 4.4 Retinoids and apoptosis of SMCs

Apoptosis of SMCs may influence the formation of intimal hyperplasia and plaque evolution \textsuperscript{177,178}. The size of the SMC population in atherosclerotic and restenotic lesions relies on the balance between cell growth and apoptosis \textsuperscript{178}. Retinoids can induce apoptosis in some cancer cell lines \textsuperscript{179} as well as fibroblasts via the Fas-FasL system \textsuperscript{180}. Similar effects have also been seen in vascular SMCs both \textit{in vitro} \textsuperscript{181,182} and \textit{in vivo} \textsuperscript{157}. Ou and co-workers suggested that the increased apoptosis was due to increased expression of tissue-transglutaminase \textsuperscript{181}, a protein involved in the formation of apoptotic bodies. Indeed, tissue transglutaminase inhibitors blocked the retinoid-induced apoptosis of SMCs \textsuperscript{181}. Interestingly, intimal SMCs have been shown to be more susceptible than medial SMCs to retinoid-induced apoptosis \textsuperscript{182}. In conclusion, retinoids inhibit proliferation and stimulate apoptosis of SMCs, preferentially of intimal origin with suggested high proliferation rate \textsuperscript{183}, which may decrease intimal hyperplasia after endovascular interventions.

### 4.5 Retinoids and SMC differentiation

Retinoids are used clinically in the treatment of disease processes involving cell hyperproliferation and dedifferentiation such as psoriasis and cancer \textsuperscript{184,185}. As discussed above, phenotypic switch from a contractile to a synthetic SMC phenotype is a central process in the response to injury to the vessel and is associated with decreased expression of differentiation markers. Retinoids have been shown to increase the expression of the SMC differentiation markers SM myosin heavy chain \textsuperscript{154}, \(\alpha\)-SM actin \textsuperscript{154,166,186-189} and others. Though most reports show increased expression of \(\alpha\)-actin after retinoid treatment, contradictory data do exist. We observed, in collaboration with Neuville \textit{et al}, decreased expression of \(\alpha\)-actin in intimal SMCs, and no effect on medial cells, after retinoid treatment \textsuperscript{167}. However, Neuville \textit{et al} showed that retinoids induce the transition from the epithelioid shape to the spindle one \textsuperscript{87}, which is generally believed to be associated with increased...
expression of SM differentiation markers. However, the data on differentiation *in vivo* is limited. Endogenous retinoic acid signaling has been shown to colocalize with the expression of the adult smooth muscle myosin heavy chain during development of the ductus arteriosus\(^{190}\). Furthermore, the level of \(\alpha\)-actin has been shown to be reduced in SMCs from vitamin A deficient rats compared to controls\(^{191}\). One definition of fully differentiated SMCs is the ability of SMCs to respond to contractile agonists. Wright and colleagues showed that the contractility of aortic SMCs could be restored in aortic rings when incubated with all-trans RA\(^{192}\). A more recent report from the same group showed reduced contraction of aortic rings from vitamin A deficient rats\(^{191}\). Thus, it seems that retinoids are involved in maintenance of a contractile, differentiated SMC phenotype, which may, together with increased ECM integrity, limit the proliferative response of SMCs after vascular injury.

4.6 **Retinoids and the fibrinolysis/coagulation system**

The homeostasis in the vessel wall includes a delicate balance between coagulation and fibrinolysis. However, pro-coagulant processes are activated at sites of vascular injury, which may lead to thrombus formation. Retinoids induce expression of tPA both in cultured SMCs\(^{167}\) and *in vivo*\(^{193-195}\) as well as in patients during retinoid treatment\(^{196}\). This induction is likely a direct transcriptional effect since the presence of a RARE in the promoter of tPA gene is seen\(^{162}\). As mentioned above, retinoids also increase the expression of PAI-1 in vascular SMCs, which may limit the proteolytic actions of tPA in the vessel wall\(^{163}\). However, the expression of PAI-1 in endothelial cells is not influenced by all-trans RA\(^{197}\). In contrast to the increased fibrinolytic activity, retinoids also decrease the coagulant properties of the vessel wall both *in vitro*\(^{198}\) and *in vivo*\(^{199}\). Retinoids have been shown to inhibit tissue factor, a strong pro-coagulant factor in the vessels\(^{199}\). Furthermore, thromboxane A2, an important inducer of platelet aggregation and vasoconstriction, is suppressed by retinoids in vascular SMCs\(^{200}\). Taken together, retinoids may prevent thrombosis through increased fibrinolytic pathways as well as decreased pro-thrombotic processes, which is of importance in preventing thrombus formation at sites of vascular injury.
4.7 Retinoids and vascular inflammation

Inflammation has a pivotal role in many vascular diseases such as atherosclerosis. As discussed earlier, most cells in the vascular wall, including SMCs, are competent to produce pro-inflammatory cytokines. Although retinoids are known to modulate inflammation, data on retinoids and vascular inflammation is limited. Retinoids may target vascular inflammation through multiple mechanisms involved in both the innate response as well as the adaptive immune response. The importance of retinoids in vascular inflammation is shown in Vitamin A-deficient rats, which have increased inflammatory response \(^{201}\). Furthermore, patients with POEMS syndrome, a disorder associated with increased circulating levels of pro-inflammatory cytokines, showed decreased levels of IL-6, TNF and IL-1\(\beta\) when treated with retinoids \(^{202}\). Additionally, a synthetic retinoid agonist was shown to decrease LPS-induced IL-6 expression in mice but did not change the TNF or IL-1\(\beta\) production \(^{203}\).

CRP is a systemic marker for inflammation and a risk factor for cardiovascular events \(^{204}\). CRP is regulated by IL-6 and contributes to complement activation and inflammation. As mentioned above, retinoids decrease IL-6 production, possibly through transcriptional cross-talk with NF-IL6 \(^{149}\). Retinoids may also influence CRP production, since an inverse relationship between plasma retinol and CRP has been suggested \(^{205}\).

The complement system plays an important role as an effector mechanism in the early phase of host defense against infections. Its activation proceeds in a sequential cascade-like fashion, resulting in production of cleavage fragments that enhance the inflammatory response and initiate formation of the cytolytic C5b-9 complex. To avoid the dramatic and injurious consequences of uncontrolled activation, the system is strictly regulated. Factor H, which participates in the control of complement activation, contains a RARE in the promoter region, and increased expression was seen in retinoid treated fibroblasts \(^{206}\). Furthermore, complement receptor type 1 (CR1), a negative regulator of the complement cascade, is increased by retinoids in hematopoietic cell lines \(^{207}\).

Retinoids decrease the expression of the vascular adhesion molecule (VCAM-1) on activated endothelial cells \(^{147}\), thereby decreasing attachment and infiltration of monocytes and T-cells into the vascular wall. Monocyte-derived macrophages are powerful producers of pro-inflammatory cytokines, which activate other immune
cells, as part of the adaptive immune response, to secrete immuno-modulatory compounds. Retinoids decrease the expression of pro-inflammatory cytokines such as TNF and IL-12 in stimulated macrophages. In contrast, RA has been shown to induce IL-1β expression in phorbol myristate acetate-activated monocytes and simultaneously inhibit the expression of IL-1 receptor antagonist. However, it seems that this induction is depending on experimental setup, since IL-1β activated monocytes was shown to have decreased expression of IL-1β, IL-8 as well as IL-6 in this cell type. IL-12 is the principle cytokine to promote T-helper 1 cells (Th1), an important conductor of the adaptive immune response. In general, T-cells of Th1 subtype are considered pro-inflammatory with its counterpart Th2, which possess anti-inflammatory effects in the vascular wall. IFNγ and TNFα are considered Th1 cytokines whereas IL-4, IL-5 and IL-10 are addressed Th2 cytokines. Retinoids have been shown to generate a Th1 to Th2 switch with decreased expression of Th1 cytokines and increased expression of Th2 cytokines in vitro and improve the clinical course in experimental allergic encephalomyelitis, which was associated with increased IL-4 production. Furthermore, vitamin A-deficient mice established a regulatory T helper cell imbalance with excess Th1 and insufficient Th2 function and had increased IFNγ production compared to controls. A third type of Th cells, Th3 cells, produces TGFβ, a powerful anti-inflammatory molecule that balances the pro-inflammatory actions in injured/activated vessels. Interestingly, induction of TGFβ and its receptor was seen in all-trans RA treated human neuroblastoma cell lines as well as in bovine endothelial cells. However, ligand-activated retinoid receptors have been shown to inhibit the AP-1 activated TGFβ-promoter. In conclusion, inflammation is a culprit process in many vascular diseases such as atherosclerosis and septic shock. Retinoids have been shown to modulate the inflammatory process in both the innate as well as the adaptive immune response through multiple mechanisms, which may be of importance in vascular diseases.

4.8 Retinoids and atherosclerosis
As discussed above, retinoids influence a vast number of processes such as proliferation, migration, differentiation, inflammation and coagulation, all of which impinge on atherosclerosis. However, the role of retinoids in atherosclerosis has retained very little attention. This could be due to the fact that several clinical trials
have evaluated the effect of β-caroten, the dietary pre-form of retinoids, with no effect on cardiovascular events, or even harmful ones in some subgroups such as smokers\textsuperscript{221,222}. However, a recent report from Dwyer \textit{et al.} showed that intima-media thickening was inversely related to plasma levels of oxygenated carotenoids such as retinol, suggesting a protective role of these compounds in early atherogenesis\textsuperscript{223}. However, one major limitation in these trials is that none has measured the plasma concentration of active retinoid ligands. In these studies, β-caroten was used in the context of their anti-oxidant capacity and their potential in gene regulation was not addressed. So far, no clinical trial has been performed with biologically active retinoids.

No significant association between plasma levels of carotenoids and retinol and the risk of vascular events due to atherosclerotic diseases has been observed\textsuperscript{224,225}. However, it is difficult to extrapolate the plasma levels of carotenoids and retinol to the availability of active retinoid ligands in the local environment of the atherosclerotic lesion.

Wuttge \textit{et al.} showed presence of the retinoid acid receptor alpha and gamma in macrophages and foam cells and presence of active retinoid ligands in atherosclerotic plaques. Furthermore, they showed increased expression of CD36, a scavenger receptor for oxidized low density lipoprotein (oxLDL), and increased uptake of oxLDL in THP-1 cells treated with \textit{all-trans} RA\textsuperscript{226}. Collectively, these preliminary data suggest that retinoids increase foam cell formation but in contrast, according to the above, reduce inflammation and possibly promote a more stable plaque. However, additional research is warranted in aspects of retinoids and atherosclerosis.
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**Table 1.** Summary of retinoid-regulated genes in vascular SMCs discussed in this thesis

Abbreviations: rSMCs: rat SMCs; haSMCs: human aortic SMCs; ceSMCs: chick embryonic SMCs
Aims of this thesis

The general objective of this thesis was to clarify the effects of retinoids in vascular injury and inflammation with special focus on vascular SMCs.

The specific aim was to investigate:

1. The molecular mechanism behind the inhibitory effect of all-trans retinoic acid on iNOS expression in vascular SMCs exposed to pro-inflammatory cytokines (paper I).

2. The effect of synthetic retinoid ligands in septic shock (paper II).

3. The retinoid metabolism and endogenous retinoid ligand production in vascular SMCs exposed to pro-inflammatory cytokines (paper III).

4. The effect of retinoids on proliferation of vascular SMCs and in the prevention of neointima formation after vascular intervention (paper IV).

5. The retinoid metabolism in vascular SMCs with phenotypic heterogeneity (paper V).

6. The proliferative effect of the substrate for active retinoid ligands in vascular SMCs with phenotypic heterogeneity (paper V).
Results and Discussion

5 Retinoids and vascular inflammation (papers I and II)

Retinoids have been shown to modulate both the innate as well as the adaptive immune response as discussed in section 4.7. The importance of retinoids in inflammation is shown in vitamin A-deficient rats, which have increased inflammatory response$^{201}$. Recently, Carlsen et al. suggested that this could be related to antagonistic cross-talk with NFκB, since NFκB activity is elevated in vitamin A-deficient rats and suppressed by surplus doses of retinoic acid$^{148}$. Furthermore, it has been shown that activated macrophages from rats deficient in vitamin A produce five times more NO than those of normal rats$^{201}$, suggesting that endogenous retinoids might dampen the production of iNOS-produced NO. This kind of constitutive inhibition of NO production could be important to prevent uncontrolled bursts of NO production. Since high concentrations of iNOS-derived NO is believed to play a crucial role in vascular inflammation$^{227}$, we hypothesized that retinoids exert some of their modulatory effects of inflammation through this system. Retinoids have previously been shown to inhibit iNOS expression in other cell types such as human keratinocytes$^{228}$, adipocytes$^{229}$, macrophages$^{208}$, murine fibroblasts$^{230}$ as well as cardiac myocytes and microvascular endothelial cells$^{231}$. Furthermore, Hirokawa et al. showed decrease iNOS expression in retinoid treated IL-1β-stimulated vascular SMCs$^{232}$. However the mechanism behind this effect was not fully explored. These in vitro data are supported by decreased iNOS expression by retinoids in vivo in a model of glomerulonephritis$^{233}$ as well as in psoriatic lesions in patients after retinoid treatment$^{234}$.

5.1 Retinoids inhibit iNOS expression through the Retinoic Acid Receptor-α (paper I)

Unstimulated rat SMCs produced almost undetectable amounts of NO. However, upon cytokine stimulation with IL-1β, increased expression of iNOS was seen with high amounts of produced NO, measured as nitrite in the culture medium. Simultaneous treatment with all-trans RA reduced iNOS expression and NO production. This could be of importance to reduce the pro-inflammatory effects of
high local iNOS expression and NO production discussed in section 1.2. However, iNOS inhibition \textit{in vivo} has not been proven conclusively beneficial. This could be due to lack of specificity, since most NOS inhibitors also reduce the constitutive, protective, eNOS-derived NO production. Interestingly, Achan and colleagues have shown that all-\textit{trans} RA increases NO production by endothelial cells without affecting eNOS expression and suggested that this was due to decreased expression of an endogenous NOS inhibitor\textsuperscript{235}.

In our study, the inhibition of iNOS expression by all-\textit{trans} RA could be abolished in the presence of a RAR-antagonist, indicating a receptor-mediated inhibition. Using synthetic agonists to different RAR isotypes, this effect was shown to be mediated through the RAR\textsubscript{α}. When the cells were transfected with a plasmid construct containing the murine iNOS promoter fused to a luciferase reporter gene, a decrease in promoter activity was seen after all-\textit{trans} RA treatment, indicating that the inhibitory effect is due to transcriptional control of the iNOS gene. Retinoids are known to regulate gene expression through transcriptional cross-talk with transcription factors such as NF\textkappa B and AP-1. Since the iNOS promoter contains several binding sites for NF\textkappa B as well as AP-1, this could be a possible mechanism behind the retinoid-mediated inhibition of the iNOS transcription. Interestingly, the glucocorticoid-mediated inhibition of iNOS transcription in human cancer cell lines is due to downregulation of the NF\textkappa B activity by the ligand-activated glucocorticoid receptor\textsuperscript{236}. Similarly, the PPAR\textgamma inhibits iNOS expression by negative interaction with NF\textkappa B and/or AP-1\textsuperscript{237}. However, we were unable to demonstrate interaction between RAR and NF\textkappa B/AP-1 on the iNOS promoter using electrophoretic mobility shift assay. In conclusion, we have identified a RAR\textsubscript{α}-mediated inhibition of iNOS transcription in vascular SMCs exposed to pro-inflammatory cytokines; this may partly explain the anti-inflammatory effects of retinoids in the vessel wall.

5.2 Synthetic retinoids improve survival in a rodent model of endotoxic shock (paper II)

To investigate the effect of retinoids in systemic inflammation \textit{in vivo}, we used a model of LPS-induced septic shock in rats. We demonstrate in paper II a significant increase in survival rate of LPS treated rats that received CD2081 or CD336, synthetic RAR\textsubscript{α} agonists, compared to rats that only received LPS. The molecular mechanism behind this dramatic effect on survival is not known at present. A similar
effect was seen in endotoxemic pigs with a decrease of several manifestations of septic shock. In contrast, Devaux and colleagues showed increased mortality in rats given all-trans RA daily at high doses for five consecutive days followed by LPS administration. In addition, increased expression of IL-2, IFNγ and iNOS was seen both in plasma and various tissues. Noteworthy, high doses of retinoids may cause “retinoic acid syndrome”, which is associated with fever, respiratory distress and edema. However, using synthetic retinoids reduces the toxicity and increase the half-life of these compounds, as demonstrated in our study.

6 Retinoid metabolism in vascular SMCs (paper III & V).

As clearly shown above, vascular SMCs respond to exogenously administrated retinoid ligands. However, the knowledge of retinoid metabolism in this cell type is very limited. A thorough knowledge of retinoid metabolism may identify targets to modulate endogenous retinoid ligand production and, hence, the biological response. Due to their location, SMCs are exposed to the plasma content of circulating retinol, the pro-hormone to active retinoid ligands. Since the concentration ratio of all-trans ROH to all-trans RA is approximately 1000:1 in plasma, target cells need RA either by endogenous cellular metabolic conversion of ROH or as active ligands from extracellular sources. Despite this, nearly all studies on retinoids and vascular SMCs are restricted to exogenously administered active retinoid ligands. We therefore aimed to investigate the metabolism of retinoids and the generation of active retinoid ligands in vascular SMCs.

6.1 Vascular SMCs metabolize retinol into active retinoid ligands (paper III and V)

As previously discussed, the generation of biologically active retinoid ligands is a two-step reaction involving substrate-specific enzymes. Vascular SMCs were shown to express the retinoid metabolizing enzymes RDH-2 and RDH-5 as well as RalDH-1 on the mRNA level, suggesting that vascular SMCs are competent producers of both all-trans RA (through RDH-2) as well as 9-cis RA (through RDH-5). Previously, RDH-5 has been shown to be expressed in smooth muscle layers in larger vessels in bovine tissues and during embryogenesis in the mouse. As mentioned earlier, all-
trans RA acts through RARs whereas 9-cis RA regulate gene transcription through both RARs as well as RXRs. However, all-trans RA may isomerize into 9-cis RA and vice versa. This process has been proposed to be non-enzymatic as well as enzyme-dependent. We used radioactively-labeled retinoids and adopted a HPLC-strategy to investigate the uptake and production of active retinoid ligands in vascular SMCs. SMCs showed a rapid uptake of both all-trans ROH as well as all-trans RA that peaked within hours.

To demonstrate metabolic conversion of the substrate, ROH, into active RAR ligands in vascular SMCs, we have used both the HPLC-assay as well as a cell-based reporter assay. Briefly, the cell-based reporter assay used human chorion carcinoma, JEG-3, cells, which was transfected with three plasmids. The first, serving as a luciferase reporter gene, contained the Upstream Activating Sequence in front of a HSV-TK promoter, and the second plasmid was either a GAL-RARα, or a GAL-RXRα construct. The two latter contained the DNA-binding domain of the yeast transcription factor GAL4 fused in frame with the ligand-binding domain of either RARα or RXRα. The third plasmid was a β-galactosidase expression vector used as a control for transfection efficiency. The transfected cells were incubated with conditioned medium from cultured vascular SMCs for 24 hours after which luciferase activity was measured. Thus, the cell-based reporter assay detects retinoid signaling in the conditioned culture medium. In contrast, the HPLC assay measures the cellular content of synthesized RA. Both methods demonstrated generation of active RAR ligands in vascular SMCs. Hence, vascular SMCs produce active retinoid ligands for transcriptional control within the cell as well as may secrete synthesized retinoid ligands to nearby located cells. This concludes that SMCs are not depending on preformed active RAR ligands, the bioavailability of which is much lesser compared to the corresponding substrate.

6.2 Increased retinoid signaling in vascular SMCs by pro-inflammatory cytokines (paper III)

As discussed above, retinoids have been shown to modulate inflammation in several vascular cell types including SMCs. However, our present knowledge of whether inflammatory mediators modulate vitamin A status in vascular cells is limited.

In paper III, we demonstrate that pro-inflammatory cytokines such as IL-1β increase the expression of RDH-5, a key enzyme in the generation of active retinoid ligands.
Out of several pro-inflammatory cytokines tested, IL-1β turned out to be the most important for the induction of RDH5. IL-1β is known to be a powerful activator of a variety of genes in vascular SMCs, such as iNOS, in contrast to other cytokines, which all act in synergy with additional cytokines to activate gene transcription.  

To investigate whether the induction of RDH-5 by pro-inflammatory cytokines was associated with increased production of active retinoid ligands, a cell-based reporter assay was used (as described above) to detect retinoid signaling. When conditioned medium from cytokine-stimulated SMCs was added to the reporter cells, a 2-fold increase of RAR signaling was seen as compared to conditioned medium from unstimulated cells. The increase was more pronounced when adding the substrate for RDH-2 and RDH-5, all-trans ROH and 9-cis ROH respectively, to the cytokine-stimulated SMCs, indicating that the supply of substrates is a limiting factor. Interestingly, we have observed increased uptake of all-trans ROH in cytokine-stimulated SMCs compared to resting cells (unpublished observations). Noteworthy, vascular SMCs are exposed to relative high concentrations of ROH in plasma in contrast to low levels of preformed, biological active, retinoic acid. Although the conditioned medium from cytokine stimulated vascular SMCs was efficient in activating the luciferase construct through the GAL4-RAR construct, no activation was detected with the GAL4-RXR construct; such activation would have been expected if 9-cis retinoic acid was synthesized by RDH-5. This may be due to the fact that the GAL4-RXR system was 100-fold less sensitive than the GAL4-RAR system. When SMCs were transfected with a wild-type RDH-5 expression vector and incubated with all-trans retinol or 9-cis retinol, an activation of the GAL4/RAR construct was seen only after addition of 9-cis retinol. These results suggest that vascular SMCs are not able to efficiently isomerize all-trans retinol to 9-cis retinol, the likely substrate for RDH-5. However, after stimulation with cytokines all-trans retinol enhanced the activation of GAL4/RAR construct. This can be explained either by an induction of an isomerase by cytokines simultaneously with RDH-5, by increased nonenzymatic conversion of all-trans retinol to 9-cis retinol or by RDH-5-independent formation of RAR ligands. Furthermore, since 9-cis RA binds both RAR as well as RXR, ligand-activated RXR may also form heterodimers with other nuclear receptors such as PPARs which have been shown to have anti-inflammatory effects in the vessel wall.
These results demonstrate that SMCs stimulated with pro-inflammatory cytokines such as IL-1β increase their production of active retinoid ligands. This, in turn, may regulate the expression of genes involved in the inflammatory process within the vessel wall. It is tempting to speculate that the increased production of active retinoid ligands in cytokine-stimulated SMCs is released to nearby cells, such as adjacent vascular cells, or infiltrating ones, such as leukocytes. Our study is, to our knowledge, the first that has identified a possible link between endogenous retinoid metabolism and vascular inflammation.

6.3 Retinoid metabolism is linked to the phenotype of SMCs (paper IV)

A fine-tuned balance between the cellular retinoid binding proteins, metabolic and catabolic enzymes results either in generation of active retinoid ligands, retinylesters for storage, or polar catabolic products for cellular excretion, which differ between cell types as well as differentiation states within one cell type. Intimal SMCs after vascular injury express more CRBP-1 than cells from the media. Intimal SMCs maintain high levels of CRBP-1 in cell culture, indicating a permanent change in the metabolism of retinoids. We therefore aimed to investigate retinoid metabolism in vascular SMCs of different phenotype.

Using a high-sensitive HPLC strategy, an increased uptake of the pro-hormone, all-trans ROH, was detected in intimal SMCs compared to medial cells, which in contrast showed increased uptake of preformed all-trans RA. Notably, due to their location, intimal SMCs are exposed to ROH in micromolar concentrations in plasma, whereas medial SMCs depend on the lower concentration of diffused ROH or preformed RA in their vicinity. Intimal SMCs exhibited increased expression of RDH-5 as well as RalDH-1, key enzymes in generation of active retinoid ligands, compared to medial SMCs. In conjunction, increased production of retinoid ligands was seen in intimal SMCs. The increased production of retinoid ligands may influence gene transcription in intimal SMCs after vascular injury. This is supported by the increased expression of CRBP-1 in intimal SMCs. This is a direct transcriptional effect of RA, through binding of the RARα-RXRα heterodimer to the RARE of the CRBP-I promoter. Noteworthy, intimal SMC are exposed to inflammatory mediators such as IL-1β after vascular injury which may activate retinoid metabolism through RDH-5 induction with increased ligand production.
Furthermore, the catabolic enzyme Cyp26A1 was expressed at five-fold higher levels in medial SMCs compared to intimal cells, which may limit the transcriptional response to retinoids in this phenotype. Thus, both retinoid activation and deactivation processes are in operation, which may, at least partly, explain the different susceptibility to retinoid treatment seen in SMCs with phenotypic heterogeneity.

7 Retinoids and SMC proliferation (paper IV & V)

Intimal hyperplasia is a key process after vascular interventions as well as in transplant arteriosclerosis. A hallmark of this process is proliferation of SMCs, but it also includes several other processes such as migration, differentiation, matrix remodeling and inflammation. Despite numerous studies and clinical trials, no causal pharmacological treatment is available today. The presently used treatment to target intimal hyperplasia after endovascular interventions is stenting, which decreases the frequency of restenosis, but it still remains a clinical problem. So far, investigated therapeutic agents have targeted a limited number of pathological processes involved, which may explain the relative lack of efficiency. Retinoids are attractive candidates since they target numerous pathways involved in the narrowing of the vessel after vascular intervention.

We therefore aimed to investigate the effect of retinoids on SMC proliferation in vitro as well as in vivo in an attempt to reduce neointima formation after vascular intervention.

7.1 Retinoids and vascular SMC proliferation (paper IV & V).

Retinoids inhibit proliferation of many cell types including vascular SMCs. This inhibition seems to involve more than a single mechanism and may differ between cell types. We and others have shown decreased proliferation rates in growth factor-stimulated vascular SMCs after all-trans RA treatment. Using synthetic retinoid agonists to each retinoid receptor isotype, we showed that this inhibition was a RARα-mediated mechanism. On the protein level, vascular SMCs mainly express RARα and most biological effects are through this isotype. As stated above, phenotypic heterogeneity of vascular SMCs influences retinoid metabolism, and hence, the response to retinoid ligands. Therefore, we investigated the growth
inhibitory effect of retinoids on SMCs with different phenotype. We have shown that all-trans RA inhibits both medial and intimal SMC proliferation in vitro and that this effect is more rapid on intimal SMCs. The difference in retinoid sensitivity can be explained by a difference in growth rates of these two subsets of SMCs, since intimal SMCs have increased replication rate compared to medial SMCs. Indeed, when the SMCs reached confluence the inhibitory effect of all-trans RA was identical in medial and intimal SMCs.

Although many reports show decreased proliferation of SMCs by active retinoid ligands, almost no studies have previously examined the effect of retinol, the pro-hormone to active retinoid ligands, on SMC proliferation. As stated above, SMCs are competent to metabolize ROH into active retinoid ligands. Indeed, serum-stimulated intimal SMCs were growth-inhibited when treated with all-trans ROH in a dose-dependent manner, indicating that intimal SMCs are capable of producing active retinoid ligands in concentrations that elicit a biological response. However, the opposite effect was seen in all-trans ROH treated medial cells with a profound, fast and significant increase in proliferation. The mechanism behind this effect is not fully explored. One can speculate that it might be due to an indirect effect from a retinoid metabolite generated from enzymatic modification by Cyp 26A1, which was shown to be 5-fold more expressed in medial SMCs compared to intimal SMCs. Indeed, unpublished observations from our group show that simultaneously treatment with a Cyp 26 inhibitor abolishes the proliferative effect in ROH treated medial SMCs. In contrast to our results, Wang et al. showed no effect on proliferation when embryonic aortic SMCs were treated with all-trans ROH \(^{249}\). However, when co-cultured with bovine endothelial cells, a significant growth inhibition was seen. These discrepant findings may be due to the embryonic SMC cell line used in this study, which may have differences in the response to growth factors \(^{250}\).

7.2 Retinoids inhibit neointima formation after vascular injury (paper IV).

A number of models are used to study potential therapeutic agents to prevent intimal hyperplasia, most of which include some form of physical insult to the vessel wall such as endothelial denudation. One frequently used model is the rat balloon-injury model, which is considered to reflect early processes thought to occur in human vessels after a balloon angioplasty.
To investigate the effect of retinoids in preventing neointima formation after vascular injury, we performed an *in vivo* study in which rats received retinoids prior to a balloon injury to the carotid artery and thoracic aorta. All-\textit{trans} RA and CD 336, a synthetic RAR\(\alpha\)-agonist, were injected intraperitoneally (both at 0.5mg/kg body weight) daily for 14 days subsequent to the balloon injury. In vehicle-treated controls, extensive intima formation was observed in all injured vessels 14 days after injury. In contrast, all-\textit{trans} RA-treated rats showed a 76% reduction in the cross-sectional area of the carotid neointima. The media itself was not affected by all-\textit{trans} RA. As a consequence of the reduction of the intima, the luminal diameter was increased by 33%. CD336 showed similar but less dramatic effects. These results are in line with the observations of Miano \textit{et al}. \textsuperscript{251}, published while paper IV was under review for publication. In addition, we showed that the effect of all-\textit{trans} RA is mediated through RAR\(\alpha\). We used low doses of all-\textit{trans} RA intraperitoneally, with no macroscopically seen tissue toxicity, in contrast to Miano who used 30mg/kg bodyweight per oral administration. After the publication of these papers, several others have shown similar effects in different injury models, species and administration approaches \textsuperscript{188,189,252-254}. This indicates that the effect of retinoids in preventing neointima formation and restenosis is not specific to a particular injury model or species. SMC proliferation is one of the key processes in restenosis. Although many strategies for combating restenosis target SMC replication, none has proven beneficial in clinical trials. Therefore, retinoids may be attractive candidates since they not only inhibit SMC proliferation but also modulate processes such as migration, apoptosis, ECM remodeling as well as coagulation and inflammation.
Concluding remarks

Although established modulators of processes known to be involved in the pathogenesis of vascular diseases, retinoids are only in the beginning to be appreciated in the cardiovascular field. This thesis may contribute to the understanding of retinoids in the modulation of vascular injury and inflammation.

Retinoids and vascular inflammation

Retinoids are known modulators of inflammation and act through multiple mechanisms. We have shown inhibitory effects on the iNOS-pathway in vascular SMCs and explored the molecular mechanisms behind this inhibition. This may be of importance in preventing the pro-inflammatory effects of high local NO production at sites of vascular inflammation. Noteworthy, retinoids have been shown to increase eNOS-derived NO production, which may offer a protective mechanism in atherogenesis and vascular inflammation/injury.

In parallel with iNOS, retinoids regulate the expression of many other genes known to be activated in diseases associated with systemic vascular inflammation. Therefor, they may be useful in the treatment of septic shock. Indeed, a dramatic increase in survival was seen in a rodent model of septic shock when given synthetic retinoid ligands. However, the molecular mechanism behind this effect is not known at present and further studies are warranted.

Retinoid metabolism

To achieve biological effects of retinoids on gene transcription, active retinoid ligands are needed. However, the knowledge of generating endogenous retinoid ligands in vascular SMCs has been limited. Our new data show that vascular SMCs are competent producers of active retinoid ligands. Importantly, intimal SMCs displayed increased retinoid metabolism and, in conjunction, increased production of active retinoid ligands compared to medial SMCs. Due to the location of intimal SMCs, this could be of importance since intimal SMCs are exposed to high local concentrations of growth factors and pro-inflammatory cytokines. Furthermore, we have for the first time provided evidence that retinoid metabolism and hence production of endogenous retinoid ligands is increased by pro-inflammatory cytokines. Thus, it is tempting to
speculate that endogenous retinoid ligands may be part of an intrinsic modulatory pathway of vascular inflammation.

*Retinoids and vascular injury*

An increasing number of studies, including our own, have demonstrated substantial effects of retinoids in preventing intimal hyperplasia and restenosis after vascular intervention. This was accomplished achieved by modulating many processes involved in the response to vascular injury. Since retinoids are used clinically today in the treatment of hematopoetic malignancies and dermatological diseases, therapeutic doses, side effects and interactions with other pharmaceuticals have been defined; this is obviously an advantage when setting up future clinical trials in the treatment of restenosis. To limit the frequency of side-effects, investigators should focus on using synthetic retinoid ligands with less toxicity and/or local administration, e.g. in coated stents.

In summary, vascular injury and inflammation are complex pathological processes which involve many cellular events including cell growth/differentiation, migration, as well as coagulation and inflammation. Future successful therapeutic compounds should interfere with many of these processes rather than with one specific trait, as do most of the available treatments of today. Retinoids have been shown to regulate many of these disease-promoting processes and may therefore represent potential future candidates in the treatment of chronic vascular inflammatory diseases (atherosclerosis), intermediate acute vascular diseases (restenosis) as well as acute vascular inflammatory diseases (sepsis and septic shock).
Future Perspectives

Although retinoids have been shown to regulate many processes associated with atherosclerosis, surprisingly few have addressed retinoids in the field of atherosclerosis. First, it is warranted to investigate retinoid metabolism in atherosclerotic plaques compared to normal arteries. Second, generating knock-out mice on apolipoprotein E-deficient background and lacking retinoid receptors and/or retinoid metabolizing enzyme would help achieving knowledge whether these modulatory effects are in operation in atherosclerosis.

The therapeutically arsenal available in the treatment of septic shock is limited to symptomatical treatment, but the use of retinoids could offer a potential future strategy since they, as mentioned, both antagonize inflammatory activated transcription factors as well as regulate the expression of many inflammatory genes involved in the vascular response to invading microorganisms. However, since the molecular mechanisms responsible for the increased survival rate in retinoid treated LPS-induced septic rats are at present unexplored, further studies are warranted.

The effects of retinoids on vascular cells have almost exclusively been studied in models using exogenous retinoid ligands, and the role of endogenous retinoid ligands in the regulation of vascular inflammation has not, so far, gained much interest. An alternative to exogenous addition of active retinoid ligands is an increase in endogenous retinoid ligands by blocking Cyp 26-dependent RA catabolism. These inhibitors are available today and have been reported to increase endogenous levels of RA with effects mimicking those of RA\textsuperscript{242,255}. Obviously, through the intracellular site of action, Cyp26-inhibitors allow RA to linger inside cells, increasing its potential to modulate gene expression. In contrast, exogenously administered RA would largely remain in the extracellular space, unable to produce a biological response and may give rise to unwanted side effects. Interestingly, early clinical trials of these inhibitors in the treatment of patients with psoriasis have been successful with only mild side effects\textsuperscript{256}. It would be of great interest to further investigate these inhibitors in context of vascular inflammation as well as in the prevention of intimal hyperplasia after vascular interventions. This strategy would increase the local concentrations of active retinoid ligands in tissues with high retinoid metabolism.

In summary, retinoids target many processes implicated in the pathogenesis of vascular diseases. Their favorable effects on cell proliferation, migration as well as
their role in modulating ECM composition, fibrinolysis and inflammation make retinoids attractive candidates in the treatment of a variety of vascular diseases including restenosis, atherosclerosis and transplant arteriosclerosis. The development of new synthetic retinoid ligands or metabolism blocking agents will further expand their use in clinical applications.
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