Proper regulation of vascular smooth muscle cell (SMC) differentiation and growth is critical for vasculogenesis and the maintenance of homeostasis in the mature vessel wall. Perturbations in the molecular circuitry governing SMC differentiation and growth are thought to be of central importance in the pathogenesis of atherosclerosis, hypertension, and restenosis after procedural revascularizations. The identification of membrane-bound receptors and the delineation of their respective signaling pathways have yielded insight into the mechanisms that control SMC differentiation and growth and have provided molecular targets for therapy of vascular disease. In recent years, several nuclear receptor binding factors have been shown to regulate SMC differentiation and growth. The steroid receptor superfamily has been of particular interest in this regard. Steroid receptors are ligand-activated transcription factors that bind discrete cis elements within the regulatory regions of a growing list of target genes. This family of nuclear receptors includes the estrogen receptors, the vitamin D receptor, the peroxisome proliferator-activated receptors, and the retinoid receptors.

Retinoid Biology: A Primer

Retinoids are natural and synthetic derivatives of vitamin A (retinol). Vitamin A is an essential vitamin that must be derived from the diet through the ingestion of vitamin A–rich foods as well as foods containing the carotenoid β-carotene, which is composed of 2 molecules of retinol. The classic studies of Wolbach and Howe demonstrated neoplasm-like growth in epithelial tissues of vitamin A–deprived rats. Conversely, many aquatic species (such as shark) exhibit a paucity of neoplasms presumably because of their extraordinarily high content of vitamin A. These findings, as well as the known antioxidant effect of β-carotene, led to several prospective studies using β-carotene supplementation as a means of reducing the incidence of cancer. The findings from these studies revealed either no benefit or an actual increase in cancer occurrence with β-carotene supplementation. Similar negative results have been reported with respect to cardiovascular disease. Thus, it appears that the parent molecule of natural retinoids has essentially no therapeutic value for the prevention of cancer and cardiovascular disease and may even be harmful to specific subpopulations (eg, smokers and alcoholics). A major limitation of these studies, however, was the lack of data pertaining to circulating levels of natural retinoids, the active metabolites of β-carotene and retinol.

The discovery of all-trans retinoic acid (atRA) as the carboxylic acid form of vitamin A led to studies that ascribed virtually all of the biological effects of vitamin A to this natural retinoid. Thus, atRA plays a vital role in normal embryogenesis and in such postnatal processes as skin and epithelial homeostasis, hematopoiesis, and spermatogenesis. An important early discovery was the demonstration that atRA could promote cellular differentiation in vitro. These findings led to the use of atRA and other natural and synthetic retinoids for the treatment of cancer. Today, atRA is standard therapy for the management of acute promyelocytic leukemia and is in various phases of clinical trials for a number of other hematological and solid tumors. The common mechanisms underlying cancer and cardiovascular diseases (ie, perturbations in differentiation and growth) suggest that retinoids could also be of therapeutic value in the treatment of certain vascular diseases (see below).

atRA is a small lipophilic molecule (300 daltons) that circulates in plasma bound to albumin at a concentration of 1 to 10 nmol/L (Figure). Experimental and clinical pharma-
Effects of Retinoids on Vasculogenesis and SMC Differentiation

The effects of retinoids on vasculogenesis have been described in several species. Early studies using retinoid-deficient avian embryos revealed an important role for atRA in the development of the cardiovascular system. Retinoids play a critical role in the regulation of gene expression during vasculogenesis, with several key findings:

1. **Immediate-Early Retinoid-Response Genes**: These genes do not require de novo protein synthesis for their expression. They are typically expressed within minutes to hours of retinoid exposure and are critical for the initial stages of vasculogenesis. Examples include genes involved in cell proliferation, migration, and differentiation.

2. **Delayed Retinoid-Response Genes**: These genes require de novo protein synthesis and consequently show somewhat slower kinetics of expression. They are involved in the later stages of vessel formation, including remodeling and maturation.

3. **Heterodimerization of Retinoid Receptors**: Retinoids activate transcription by binding to retinoid receptors, which form heterodimers with the retinoid X receptors (RXRs). These heterodimers can then bind to retinoic acid response elements (RAREs) in the regulatory region of many genes, leading to expression of target genes.

4. **Regulation of Vascular Homeostasis**: Retinoids can also modulate the expression of genes involved in vascular tone, remodeling, and inflammation, playing a crucial role in maintaining vascular homeostasis.

5. **Clinical Relevance**: The effects of retinoids on vasculogenesis have been implicated in the pathogenesis of various vascular disorders. For example, disruptions in retinoid signaling can lead to abnormalities in vessel formation and function, which may contribute to conditions such as atherosclerosis and diabetic retinopathy.

These findings underscore the importance of retinoids in the development and maintenance of the cardiovascular system, highlighting the potential for therapeutic interventions targeting retinoid signaling in the treatment of vascular diseases.
in the establishment of an intact intraembryonal-extraembryonal circulatory network; in the absence of atRA, there was no vitelline artery or omphalomesenteric vein, and the embryos died.45,46 Mice homozygous null for retinaldehyde dehydrogenase-2, a key enzyme for atRA synthesis, display severe extraembryonic vascular defects and die at midgestation.47 These studies indicate that low or absent levels of atRA have profound consequences for normal vascular development. Conversely, excess atRA induced an avascular yolk sac in mouse embryos through a protease-mediated reduction in basic fibroblast growth factor expression.48 Moreover, administration of 13-cis RA to pregnant humans resulted in a RA embryopathy characterized by malformations of the great vessels.49 Thus, levels of retinoids in developing embryos appear to be of critical importance for proper vasculogenesis to proceed.

More direct evidence implicating atRA in vasculogenesis is offered from studies in retinoid receptor knockout mice. With the exception of RXRα null mice, which show hypoplastic thinning of the developing myocardium leading to midgestation lethality,50,51 none of the single–retinoid receptor knockout mice shows discernable defects in the cardiovascular system. Instead, the remaining single–retinoid receptor null mice show perinatal lethality as a result of a generalized growth deficiency (RARα⁺, RARγ⁺), sterility (RARα⁺, RARγ⁺, and RXRβ⁺), or essentially no phenotype (RARβ⁺ and RXRγ⁺).52,53 On the other hand, compound retinoid receptor knockout mice (eg, RARα⁻/RXRα⁻) exhibit multisystem defects and vascular malformations, including a persistent truncus arteriosus; absence of the stapedial artery (second aortic arch derivative); and alterations in the third, fourth, and sixth aortic arches.52,54 Importantly, no studies to date have examined SMC differentiation directly in compound retinoid receptor knockout mice. Such an examination is now possible with the generation of several transgenic mouse lines carrying the lacZ reporter gene under control of smooth muscle (SM)–restricted promoters.55–57

<table>
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<tr>
<th>Gene</th>
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<th>Mechanism of Gene Expression</th>
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<td>PKCε</td>
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<td>Bcl-2</td>
<td>↓</td>
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Italicized genes represent those of which the modulation by retinoids has been documented in vascular SMCs. Most of the genes induced with retinoids harbor one or more RAREs in their regulatory region and can largely be classified as early retinoid-response genes (an exception is the laminin B1 gene). Most non–RARE target genes require de novo protein synthesis for the activation or suppression of retinoid-mediated gene expression and, hence, are referred to as delayed retinoid-response genes (an exception here is the AT1 receptor). Mφ indicates macrophage; NRE, negative RARE.
If, on the other hand, the colocalization of activated retinoid receptors and SM2 is unique to the ductus arteriosus, regional variations in the concentration and/or metabolism of atRA as well as the repertoire of expressed retinoid receptors could explain such specificity. Further exploration of this intriguing finding, including a thorough examination of expression of the RARE-lacZ reporter in adult mice combined with other SMC markers, is warranted.

A number of in vitro studies have shown that atRA can positively influence the SMC differentiation program. Hayashi et al. showed that atRA stimulated the expression of tropoelastin mRNA and the subsequent elaboration of elastin in chick embryonic vascular SMCs. Haller et al. found that atRA promoted the differentiation of primary-derived rat aortic SMCs as assessed by an increase in protein kinase C (PKC)–α and SM α-actin expression. When PKCα was microinjected into growing SMCs, there was a corresponding increase in SM α-actin immunostaining. The nature of the signaling pathway involved in atRA-mediated PKCα activation and subsequent SM α-actin expression is unclear at this time. However, induction of PKCα by atRA is likely to occur at the level of transcription through a consensus DR-5 RARE in the 5′ promoter of PKCα. In the P19 embryonic cell model system of SMC differentiation, atRA was shown to stimulate several SMC markers, including SM α-actin, SM myosin heavy chain, and the α7 integrin receptor. An elevation in the expression of SM α-actin and SM myosin heavy chain was also observed in atRA-treated embryonic stem (ES) cells. Because undifferentiated P19 cells and ES cells represent multipotential cell lineages, induction of highly restricted SMC gene markers in these cells by atRA suggests that the hormone may stimulate a subset of transcription factors that orchestrate a program of SMC differentiation. In fact, atRA treatment of P19 cells stimulates the expression of MHox, a homeodomain-containing transcription factor that potentiates the expression of SM α-actin. It is possible that other atRA-inducible homeobox genes function in a similar context (Table).

A major criterion for defining a fully differentiated SMC is the ability of SMCs to respond to contractile agonists by increasing intracellular calcium, activating the myofilament apparatus, and generating force. Blank et al. showed significant increases in intracellular calcium after G protein–coupled receptor activation of a SMC clone derived from atRA-treated P19 cells; parental (non-atRA-treated) P19 cells showed essentially no such elevation in intracellular calcium. ES cells can spontaneously differentiate into contracting SMCs in normal medium; however, the frequency of contracting SMCs increases dramatically in the presence of atRA. Using patch-clamp methods and ion-channel inhibitors, both a calcium-activated maxi-K⁺ channel and a delayed rectifier K⁺ current were established in SMCs derived from atRA-treated ES cells. Gollasch et al. demonstrated expression of a class C L-type calcium channel in atRA-stimulated A7r5 SMCs. Interestingly, expression and activity of the L-type calcium channel appeared to correlate with expression of SM α-actin and SM myosin heavy chain, suggesting that ion channels and contractile elements are coordinately regulated by atRA. Whether atRA itself can provoke SMC contraction in these in vitro model systems remains to be investigated. However, Wright et al. showed that the “contractile competence” of aortic SMCs could be restored if aortic rings were incubated in medium containing atRA. Plasma proteins (probably albumin) were necessary for the ability of atRA to restore contractile activity of organ-cultured aortic rings. Taken together, there is considerable evidence supporting an important role for atRA in the maintenance and possible establishment of a SMC-differentiated phenotype.

Effects of Retinoids on SMC Growth

Retinoids have variable effects on SMC growth modulation in vitro, depending on study design. For example, in the absence of growth factor, retinoids may stimulate SMC proliferation, possibly through the activation of cyclins D and E. On the other hand, there are reports of retinoids directly inhibiting or having no effect on SMC proliferation. Variations in the species of SMCs, the concentration, and/or type of retinoid used, as well as its source, may contribute to such disparate findings. In contrast to these variable findings, several studies have shown retinoids to attenuate growth factor–stimulated SMC proliferation. An early report showed that atRA suppressed platelet-derived growth factor-BB–stimulated SMC proliferation in human intimal SMCs. Similarly, atRA and 9-cis RA blocked platelet-derived growth factor-BB–induced rat aortic SMC proliferation. Chen and Gardner showed that several retinoids reduced the growth-stimulatory effects of endothelin (ET) in rat aortic SMCs. A corresponding decrease in ET-induced extracellular signal–regulated kinase (ERK) activity was also observed in retinoid-treated SMCs. The inhibition by atRA of ET-induced ERK activity appeared to be mediated by retinoid receptors, as 2 synthetic retinoids that selectively activate RARs (TTPNB) or RXRs (LG110153) also blocked ET-mediated ERK activation. In the same report, atRA was shown to stimulate p21 promoter activity and upregulate the endogenous p21 protein, a potent inhibitor of cell cycle progression in SMCs. More recently, several retinoids were shown to inhibit serum- and serotonin-induced canine SMC growth. RARγ-selective retinoids were more effective in inhibiting serotonin-induced SMC proliferation than other retinoid receptor agonists. Although RARγ has been reported to be restricted to lung and skin, SMCs express high levels of RARγ mRNA.

Retinoids have been shown to modulate endothelial cell growth and shape in vitro. Moreover, treatment of endothelial cells with retinol resulted in the elaboration of a SMC growth inhibitor that was released into the endothelial cell culture medium. Endothelial cells are known to express cellular retinol binding protein, which facilitates the metabolism of retinol to atRA. Thus, one mechanism for medial SMC growth suppression may be via endothelial cell–derived atRA generation from plasma retinol. It should be noted that cultured endothelial cells metabolize atRA in a cytochrome P-450–dependent manner, which has consequences for the availability of atRA to SMCs of the vessel wall (see below).
Effects of Retinoids on Neointimal Formation

In the last 3 years, several papers have emerged showing that retinoids are effective in promoting a larger luminal area after mechanical injury to the vessel wall. Miano et al. showed that atRA attenuated neointimal formation in the rat carotid artery model. An elevation in intravascular retinoid levels verified that biologically active atRA was in close proximity to the medial SMC population. Decreases in intimal area coincided with a significant reduction in intimal cell number, suggesting that atRA interfered with SMC proliferation and/or migration. Support for growth inhibition was obtained with bromodeoxyuridine studies showing decreased medial (but not perivascular) SMC DNA synthesis in animals treated with atRA. It is also possible that atRA stimulated an exaggerated apoptotic response to injury. Neuville et al. demonstrated similar inhibitory effects of atRA on neointimal formation and provided evidence for the involvement of active retinoid receptors in this process. DeRose et al. used both atRA and 13-cis RA to inhibit neointimal formation and SMC DNA synthesis in the rat carotid artery model. The advantage of using 13-cis RA is its longer half-life and lower toxicity profile as compared with those of atRA. Chen et al. showed that atRA blocked both neointimal formation and medial SMC DNA synthesis in a rabbit iliac artery model of restenosis. In a very recent report, Lee et al. found that atRA attenuated neointimal formation and accelerated reendothelialization in the balloon-injured rat aorta. Importantly, all of the above in vivo studies involved near-uniform removal of the endothelium. Because endothelial cells can metabolize atRA, possibly limiting the bioavailability of atRA to underlying mural cells of the media and adventitia, it will be important to ascertain whether atRA can inhibit lesion development in the context of an intact endothelium. Studies using atRA administration in a flow-reduced model of neointimal formation (where the endothelial cell monolayer is not removed) may help to address this important issue.

How might retinoids impede neointimal formation? A likely mechanism is through retinoid receptor–mediated changes in gene expression. As pointed out above, there is evidence that atRA partially blocks SMC proliferation in vivo. Several genes involved with growth regulation could be targets of activated retinoid receptors (Table). An equally plausible mechanism may be through modulated expression of genes involved with SMC migration. For example, studies performed in vitro show that atRA reduces collagenase expression in human intimal and rabbit aortic SMCs with a concomitant inhibition in migration. The inhibitory effect of atRA on collagenase expression appears to be at the level of the promotor. The collagenase promoter is regulated, in part, by the activated protein-1 (AP-1) complex of Fos and Jun family members. Activated retinoid receptors antagonize AP-1–dependent gene transcription through the sequestration of coactivators, which are critical for AP-1 to mediate gene transcription. It should be mentioned that although there is evidence for retinoid-mediated inhibition of SMC migration, there have been 2 reports showing the opposite effect. Clearly, more work is necessary to clarify these differential effects of retinoids on SMC migration.

As with collagenase, transforming growth factor–β1 (TGF-β1) gene expression is dependent on AP-1 activity and hence is susceptible to the antagonistic effects of atRA. Expression of TGF-β1 is elevated after balloon injury and may be involved in neointimal formation through its effects on SMC growth, migration, and extracellular matrix accumulation. Whether atRA interferes with collagenase, TGF-β1, or other SMC AP-1–dependent genes in vivo is not known. In fact, the full repertoire of AP-1–dependent genes that are expressed in activated SMCs has yet to be defined. Because retinoids are potent antagonists to AP-1–dependent gene transcription, identifying all of the AP-1–regulated genes in SMCs may yield mechanistic insight into the action of retinoids within the vessel wall. Of course, we cannot discount nongenomic effects of retinoids such as retinoylation (a post-translational modification of mature proteins leading to their inactivation) as a mechanism for the inhibitory effects of atRA on neointimal formation.

In addition to inhibiting neointimal formation, retinoids appear to exert effects on vessel remodeling. For example, atRA promoted outward remodeling of the balloon-injured rat carotid artery. In an independent study using the same model, an inhibition of inward remodeling was observed. Recently, Wiegman et al. showed that atRA effected outward remodeling of the femoral artery in an atherosclerotic rabbit model of restenosis. The latter study failed to detect decreases in intimal area or SMC DNA synthesis; however, significant increases in SM α-actin and desmin were observed in atRA-treated vessels, suggesting that SMC phenotype may be an important determinant of the remodeling that ensues with atRA treatment. Collectively, the in vivo data thus far are consistent in showing that retinoids exert desirable changes in vascular geometry after injury.

Future Directions

The fields of retinoid biology and vascular SMC biology have converged with the generation of many important observations pertaining to the pathobiology of vessel wall diseases. Several mechanistic issues surrounding retinoid action in SMCs and the vessel wall will require detailed experimentation. First, it will be very instructive to identify all retinoid-responsive genes in SMCs to direct future studies aimed at understanding retinoid action on SMC differentiation and growth processes. Moreover, identifying SMC retinoid-response genes will likely illuminate in vivo pathways involved with vasculogenesis and the inhibition of neointimal formation. Second, the relationship between retinoids and SMC apoptosis in the vessel wall needs attention, particularly given that retinoids are known to induce apoptosis in the setting of cancer. Third, the availability of retinoid receptor knockout mice will allow for the study of retinoid-mediated effects on cultured SMCs, as well as vessel wall injury responses in defined genetic backgrounds in which 1 or more retinoid receptors have been inactivated. These mouse models should also be crossed with SMC promoter mice (eg, SM22-lacZ) to assess the role of individual retinoid receptors or combinations therein on vascular SMC differentiation. Fourth, the growing number of synthetic retinoids should be exploited to carefully dissect out which retinoid receptors...
mediate changes in SMC biology. Indeed, several studies have already begun such an analysis.69,78,83,94,95 Finally, given the multifactorial and unpredictable nature of vascular diseases and the pleiotropic effects of retinoids, an evaluation of retinoid efficacy in the setting of vascular disease should be considered, particularly given that several retinoids have already been approved for clinical use.

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References


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