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Novel cardiovascular actions of the activins

C J Molloy¹, D S Taylor² and J E Pawlowski³

¹3-Dimensional Pharmaceuticals, Inc., Exton, Pennsylvania 19341, USA

²Bristol-Myers Squibb Pharmaceutical Research Inst., Princeton, New Jersey 08543, USA

³University of Colorado Health Sciences Center, Denver, Colorado 80262, USA

(Requests for offprints should be addressed to C J Molloy, 3-Dimensional Pharmaceuticals, Inc., 665 Stockton Drive, Suite 104, Exton, Pennsylvania 19341, USA)

Abstract

Proliferation and directed migration of vascular cells are key components in vascular diseases such as atherosclerosis and restenosis following percutaneous transluminal coronary angioplasty. However, the precise cellular and molecular mechanisms involved in the control of vascular cell proliferation or migration at the tissue level remain largely undefined. Molecules contributing to these processes are elaborated by distinct cell types and act in both autocrine and paracrine modes. They include two broad classes, polypeptide growth factors and vasoactive G-protein-coupled receptor (GPCR) agonists. Examples of the former, such as platelet-derived growth factor, bind to and activate cell surface receptor tyrosine kinases, initiating intracellular biochemical signaling pathways associated with cell proliferation or migration. In contrast, recent evidence suggests that vasoactive GPCR agonists (e.g. angiotensin II, endothelin-1, α -thrombin) elicit cell growth indirectly by inducing the production of autocrine or paracrine factors in vascular cells. Recent studies have

identified activin A as a novel component of conditioned medium obtained from GPCR agonist-stimulated vascular smooth muscle cells (SMCs). Although activin A alone only weakly stimulated rat aortic SMC DNA synthesis, it demonstrated a potent co-mitogenic effect in combination with either epidermal growth factor (EGF) or heparin binding EGF-like growth factor in these cells, increasing DNA synthesis by up to 5- and 4-fold respectively. Furthermore, in a rat carotid-injury model, activin A mRNA was upregulated within 6 h after injury, followed by increases in immunoreactive protein detected in the expanding neointima 7 to 14 days later. Taken together, these results indicate that activin A is a common vascular SMC-derived growth factor induced by vasoactive agonists that may, either alone or in combination with other factors, contribute to fibroproliferative vascular diseases.

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Introduction

Excessive proliferation and migration of several vascular cell types (e.g. vascular smooth muscle cells (SMCs), endothelial cells, myofibroblasts) are important components in vascular remodeling and associated pathologies, including angiogenesis, atherosclerosis, and restenosis following percutaneous transluminal coronary angioplasty (PTCA) (Schwartz *et al.* 1990, Ross 1993, Schwartz 1997). Accordingly, in normal vessels, cell growth and migration are closely regulated processes, although the precise molecular mechanisms involved in each are not well understood.

In the mature artery, complex interactions between the different resident cells, as well as circulating cells, influence the growth and metabolic rates of individual vascular cell

types. Thus a balance of paracrine growth factors, vasoactive factors, circulating hormones, etc. controls tissue homeostasis. For example, endothelial cells lining intimal arterial surfaces produce nitric oxide and other factors that act to reduce vascular SMC proliferation (Gibbons & Dzau 1994). Consequently, endothelial cell damage, initiated either by direct injury or the accumulation of inflammatory cells, may alter the expression of these factors resulting in enhanced proliferation and/or migration of vascular SMCs involved in neointimal formation. These events contribute to the process of atherosclerosis, resulting in the formation of inflammatory fibroproliferative lesions that ultimately lead to myocardial and/or cerebral ischemia. Hence, a fundamental challenge in vascular pathobiology is the identification of the critical endogenous factors involved in vascular responses to injury.

Figure 1 Autocrine model of delayed mitogenesis stimulated by G-protein-coupled receptor agonists in vascular SMCs. Binding of vasoactive factors to the appropriate receptor results in rapid intracellular signal transduction leading to the expression of endogenous mitogenic factors (see text). The secretion of one or more mitogenic factors results in the formation of a productive autocrine loop, leading to cellular proliferation. Therapeutic agent(s) targeted against a key autocrine mitogen (growth factor (GF) antagonist) may functionally antagonize several GPCR agonists. ET-1, endothelin-1.

Several G-protein-coupled receptor (GPCR) agonists including angiotensin II (AII) and α -thrombin have been implicated in vascular pathologies. These proteins are potent mitogens for vascular SMCs *in vitro* (Scott-Burden *et al.* 1991, McNamara *et al.* 1993, Weber *et al.* 1994) and also induce extracellular matrix formation (Scott-Burden *et al.* 1990, Carney *et al.* 1992). AII acts by direct activation of the angiotensin type 1 receptor (Sung *et al.* 1994), whereas α -thrombin binds to its receptor and, by enzymatic cleavage generates a new amino-terminus 'tethered ligand' which activates the receptor (reviewed in Coughlin 1994). Early intracellular signaling events in rat aortic smooth muscle (RASM) cells in response to AII and α -thrombin include increased levels of intracellular Ca^{2+} , phosphoinositide turnover and formation of diacylglycerol (Berk *et al.* 1991, Matsusaka & Ichikawa 1997). AII and α -thrombin also stimulate signaling events associated with cellular proliferation including protein tyrosine phosphorylation, activation of the Raf-1/mitogen-activated protein kinase pathway, and nuclear proto-oncogene expression (for reviews see Van Obberghen-Schilling & Pouyssegur 1993, Duff *et al.* 1995).

Many of the mitogenic signaling events activated by AII and α -thrombin are shared with growth factor agonists of receptor tyrosine kinases. However, the induction of DNA synthesis in response to AII or α -thrombin in vascular SMCs is delayed when compared with mitogens such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF) or fetal bovine serum (Weber *et al.* 1994, Molloy *et al.* 1996). This delay may be due to a requirement for the production of endogenous

growth factors in vascular SMCs that mediate the GPCR agonist-mediated responses. In this regard, AII and α -thrombin have been shown to stimulate the expression of growth factors including PDGF-A chain (Naftilan *et al.* 1989, Stouffer *et al.* 1993), basic FGF (Itoh *et al.* 1993, Weiss & Maduri 1993) and insulin-like growth factor (IGF)-I (Delafontaine *et al.* 1996) which may contribute to the mitogenic effects of these GPCR agonists. Taken together, these data support the hypothesis that various peptidic, GPCR agonists each stimulate vascular SMC proliferation by a similar autocrine mechanism (Fig. 1). In this model, delayed mitogenesis is preceded by the increased expression of a common subset of new gene products in vascular SMCs. Work from our laboratory, described below, has identified activin A as one of these common gene products upregulated by vasoactive GPCR agonists.

Identification of activin A in conditioned media samples from GPCR agonist-stimulated vascular SMCs

Our laboratory had previously demonstrated that RASM cells treated with the GPCR agonists α -thrombin or AII secreted potent mitogenic factors into the cell culture media (Weber *et al.* 1994, Molloy *et al.* 1996). Since previous studies had implicated growth factors such as basic FGF, PDGF, or IGF-I in this mechanism, we attempted to identify the mitogenic components in GPCR agonist-stimulated RASM cell conditioned media

using a panel of specific growth factor-neutralizing antibodies. However, none of the antibodies significantly inhibited the mitogenic activity present in the RASM cell conditioned media samples. During these experiments we observed one notable exception. An individual lot of purified polyclonal antibodies (AD921221), obtained from a rabbit immunized with chromatographically-purified human placental basic FGF, was found markedly to inhibit the RASM cell-secreted mitogenic activity (Pawlowski *et al.* 1997). Since we previously had excluded basic FGF as a contributing mitogen, immunoblot analysis was performed on concentrated samples of conditioned media obtained from thrombin- or AII-stimulated RASM cells to determine whether these 'neutralizing' antibodies cross-reacted with another SMC-derived mitogen. SDS-PAGE and immunoblot analysis identified a protein of 14 kDa under reducing conditions (~26 kDa under non-reducing conditions) which was not recognized by other anti-basic FGF antibodies. This protein subsequently was purified and identified as activin A (inhibin β_A homodimers; for a review see Vale *et al.* 1990) by Edman sequencing and mass spectroscopy analysis. The reason for the cross reactivity of the anti-basic FGF antibodies with activin A is still unclear; however, these results demonstrated that activin A expression was strongly upregulated by GPCR agonists, but only weakly induced by PDGF or serum. Phorbol 12-myristate,13-acetate also stimulated activin A expression, suggesting a requirement of the protein kinase C pathway for this effect (Pawlowski *et al.* 1997).

Northern analysis using a probe complementary to the activin A coding region was used to determine whether the expression of activin A was regulated at the mRNA level in RASM cells treated with AII. Since activin A is produced as a pro-peptide that is proteolytically processed to the mature form, enhanced expression of activin A protein from stimulated vascular SMCs could be due to increased levels of mRNA transcription, increased proteolytic processing, or both. Northern analysis demonstrated that activin β_A mRNA levels increased within 3 h of AII stimulation, peaked at approximately 6 h following treatment and returned to basal levels by 18 to 24 h. Although the effects of AII on protein translation and post-translational processing were not investigated, these experiments indicated that AII significantly increased the activin A mRNA levels in cultured RASM cells.

Mitogenic activity of activin A towards vascular SMCs

Recombinant activin A was used to confirm its mitogenic activity towards vascular SMCs. In these experiments, we observed that activin A (20 ng/ml) stimulated only a modest 50% increase in [3 H]thymidine incorporation compared with control cells (Pawlowski *et al.* 1997). These

Figure 2 Activin A potentiates the mitogenic activity of EGF in cultured vascular SMCs. Quiescent RASM cells were treated for 24 h with increasing concentrations of EGF as indicated in the absence (closed circles) or presence (open circles) of activin A (20 ng/ml). Results correspond to [3 H]thymidine incorporation of treated cells expressed as a percentage of the serum-starved control levels. Each condition represents the mean $\pm$ S.D. of 3 treatment wells from a representative experiment. Reprinted with permission from Pawlowski *et al.* (1997).

results indicated that activin A alone could not account for the pronounced mitogenic activity found in thrombin- or AII-stimulated vascular SMC conditioned media samples. Therefore, we explored the possibility that activin A might enhance the activity of other growth factors. In these experiments, we found that although activin A did not complement the mitogenic activity of basic FGF or PDGF, it strongly potentiated vascular SMC DNA synthesis stimulated by epidermal growth factor (EGF) (Fig. 2) or heparin binding EGF-like growth factor (HB-EGF) (Pawlowski *et al.* 1997). Furthermore, consistent with other studies, we found that EGF or HB-EGF alone were only weakly mitogenic towards vascular SMCs. Taken together, these results suggest that activin A facilitates the proliferative responses of vascular SMCs to a subset of endogenous growth factors, i.e. the EGF growth factor family.

Expression of activin A in vascular injury

To determine whether activin A expression was present in vascular injury, we measured activin A mRNA and protein levels following vascular injury using the rat carotid balloon-injury model (Pawlowski *et al.* 1997). We observed significantly increased levels of activin A mRNA by 6 h following injury, compared with uninjured vessels. However, the induction of activin A mRNA expression was transient since no increases were seen 3 or 6 days following injury. In contrast, we observed sustained and markedly upregulated expression of activin A protein

Figure 3 Increased activin A protein expression observed *in vivo* following balloon injury of rat carotid arteries. Photomicrographs (magnification $\times 40$) of carotid artery immunohistochemical specimens obtained from uninjured animals (A, B) or 14 days following balloon injury (C, D) are shown. (A) Control artery stained with anti-activin A antibodies (red reaction product). (B) Uninjured artery stained with anti- α -actin antibody (blue reaction product) identifies smooth muscle cells in the media. (C) Carotid artery 14 days after balloon injury. Note that increased anti-activin A antibody staining (red) was localized to neointimal cells as well as a few medial cells. (D) In the 14-day injured artery, anti- α -actin antibodies (blue) identified smooth muscle cells in both the media and neointima. Reprinted with permission from Pawlowski *et al.* (1997).

following carotid injury (Fig. 3). In these experiments, antibodies directed against either SMC α -actin or activin β_A chain were used to identify vascular SMCs and activin A protein respectively in rat carotid arterial specimens. In uninjured control arteries, α -actin-positive medial SMCs expressed low levels of activin A which was also faintly identified in adventitial layers (Fig. 3A). Following injury, activin A expression was strongly induced. For example, at 2 and 4 days after injury, increased staining of activin A was observed in both the media and adventitial layers, as well as the slowly accumulating intimal vascular SMCs (data not shown). Seven days after injury, increased amounts of activin A were observed in the adventitia as well as in the SMCs of the media and neointima. By 14 days, activin A staining was most pronounced in neointimal SMCs, while its staining in the adventitia and media had diminished (Fig. 3C). By comparison, SMC α -actin characteristically was detected in both medial and neointimal cells (Fig. 3D). Double immunostaining with anti-activin A and anti- α -actin antibodies revealed many cells that contained both antigens, confirming that neointimal and medial SMCs identified by α -actin staining also expressed activin A (data not shown).

Activin A expression following vascular injury: pathophysiological implications

Activin exists as disulfide-linked homodimers or heterodimers of the inhibin β_A and/or β_B polypeptide chains respectively (Vale *et al.* 1990). These proteins were originally identified by their ability to stimulate the secretion of follicle-stimulating hormone from anterior pituitary cells. However, activin A has been reported to affect many other cell types; for example, it acts as an erythroid differentiation factor (Yu *et al.* 1987), as well as a potent inducer of mesoderm in *Xenopus laevis* (van den Eijnden-van Raaij *et al.* 1990, Smith *et al.* 1990). Activin A has also been shown to induce DNA synthesis in porcine thyroid cells (Kotajima *et al.* 1995), MC3T3-E1 osteoblasts (Hashimoto *et al.* 1992) and preleptotene spermatocytes (Hakovirta *et al.* 1993).

As outlined above, activin A may function in the cardiovascular system by mediating the proliferative response of vascular SMCs stimulated with certain vasoactive GPCR agonists. Thus, although activin's mitogenic properties on vascular SMCs and fibroblasts have been described (Kojima *et al.* 1993, Sakurai *et al.* 1994), we observed that it was only weakly mitogenic when added

Figure 4 Progression of vascular fibroproliferative diseases: potential role of activin A. The progression of fibroproliferative vascular disease involves different cell types (underlined), including monocyte/macrophages, vascular smooth muscle cells (SMC), and myofibroblasts (for a review see Ross 1993). Surgical treatments in the disease process include percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG). Activin A may have a role in the regulation of vascular SMC/myofibroblast proliferation, migration, and matrix formation.

alone to vascular SMCs, but significantly potentiated the levels of DNA synthesis stimulated by EGF or HB-EGF. Although the exact mechanism for this effect awaits further investigation, it could be due to an induction of EGF receptor expression or modulation of EGF-receptor binding affinity as previously reported for transforming growth factor- β (TGF- β) and EGF (Assoian *et al.* 1984, Massague 1985). Furthermore, since EGF (Rossi *et al.* 1992, Sambhi *et al.* 1992) and related factors including HB-EGF (Temizer *et al.* 1992, Miyagawa *et al.* 1995) and epiregulin (Taylor *et al.* 1999) have been implicated in vascular SMC growth control, our results support a modulatory role for activin A in this process.

Additional support for a role of activin in the cardiovascular system derives from the observation that intimal activin A protein content was found to be markedly upregulated following arterial injury *in vivo*. These data reinforce previous studies that have identified activin A expression in arteriosclerotic lesions from hyperlipidemic rabbits (Inoue *et al.* 1994) and in the medial layer of human coronary artery samples (Kozaki *et al.* 1995). More recently, de Vries and colleagues have reported that monocytes/macrophages, as well as human vascular SMCs, express activin upon stimulation with atherogenic stimuli (C J M de Vries, personal communication). They also showed that activin A induced the expression of specific proteins associated with vascular SMC differentiation, and postulate that activins may have a role in atherosclerotic plaque stabilization as well as vascular injury repair. Activin A has also been shown to increase monocyte (Petraglia *et al.* 1991) and vascular SMC (M C Reidy & C E Turner, personal communication) migration. Taken together, these results imply that activin A may contribute to the progression of fibroproliferative vascular diseases,

including atherosclerosis and post-PTCA restenosis (Fig. 4). Thus, activin A secreted from 'injured' vascular SMCs might act upon adventitial fibroblasts, SMCs, and monocytes/macrophages to promote arterial remodeling. Furthermore, since activin A inhibits proliferation of endothelial cells (McCarthy & Bicknell 1993), it is possible that excessive activin expression in the artery wall may delay re-endothelialization at the site of vascular injury.

Activin A is a member of the TGF- β superfamily comprised of other factors that have previously been implicated in vascular diseases (Border & Ruoslahti 1992). For example, Wolf *et al.* (1994) have demonstrated that neutralizing antibodies directed against recombinant TGF- β_1 could suppress neointimal formation in the rat carotid-injury model. Additionally, other TGF- β isoforms are well-characterized stimuli for extracellular matrix protein production, in connective tissue cells (Roberts *et al.* 1986, Roberts 1995). The functions of activin A in vascular injury, therefore, may include the direct and indirect stimulation of tissue remodeling. In support of this concept, a recent study reported that the formation of embryonic cardiac cushion tissue, which ultimately contributes to formation of heart valves and septa, is dependent on the expression of activin A mRNA as measured by Northern blotting and *in situ* hybridization (Moore *et al.* 1998). Thus, localized expression of activin A in the heart suggests a possible role in the endothelial-mesenchymal transitions related to vascular remodeling.

The expression of activin A has also been correlated with fibrosis and tissue repair in other organ systems. In this regard, increased expression of activin A has been reported in rat liver fibrosis/cirrhosis (Sugiyama *et al.* 1998) and in pulmonary fibrotic lesions (Matsuse *et al.* 1996). Other studies have identified activin A expression

associated with skin wounding (Hübner *et al.* 1996). In a related report, Munz *et al.* (1998) demonstrated that transgenic expression of activin A in mouse epidermis led to skin thickening, fibrosis, and accelerated wound healing in response to epidermal injury. These results support experiments that correlated delayed wound healing with reduced activin A expression following cyclosporine A administration in a rat model *in vivo* (Petri *et al.* 1998). Taken together, these findings indicate that activin A may be an important mediator of both normal and pathological wound healing in a variety of tissues.

Conclusion

The adverse vascular remodeling that occurs in diseases such as atherosclerosis and restenosis involves the migration and proliferation of multiple cell types in the vessel wall. Despite the fact that many endogenous molecules have been reported to stimulate these processes, accumulating evidence suggests that only a subset of factors (e.g. PDGF, GPCR agonists) significantly contribute to the progression of fibroproliferative vascular diseases. Activin A has recently been identified as a secreted protein expressed by vascular SMCs in response to GPCR agonists and vascular injury. Thus, this molecule may play an important role in the control of cellular processes including proliferation, migration, differentiation and matrix formation associated with the progression of vascular lesions. Future development of therapeutic strategies to modulate the activity of activin A may lead to clinically useful treatments for the amelioration of important vascular diseases, including atherosclerosis and post-PTCA restenosis.

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