Adenoviral Activin A Expression Prevents Intimal Hyperplasia in Human and Murine Blood Vessels by Maintaining the Contractile Smooth Muscle Cell Phenotype


Abstract—Activin A alters the characteristics of human arterial smooth muscle cells (SMCs) toward a contractile, quiescent phenotype. We hypothesize that activin A may prevent SMC-rich neointimal hyperplasia. Here, we study the effect of adenovirus-mediated expression of activin A on neointima formation in vitro and in vivo. Human saphenous vein organ cultures, in which a neointima is formed spontaneously, were infected either with activin A- or lacZ-adenovirus. Activin A-overexpression reduces neointima formation by 78%, whereas no significant reduction was observed after control infection. In addition, the effect of activin A on neointima formation was assessed in vivo in mice with cuffed femoral arteries. In activin A adenovirus-infected mice (IV injection), neointimal hyperplasia is reduced by 77% compared with the SMC-rich neointima in mock-infected or in noninfected mice. Cultured human saphenous vein SMCs and murine aorta SMCs were incubated with activin A and an increased expression of SM22α and SM α-actin mRNA, and SM α-actin protein was demonstrated. Laser-capture microdissection on sections of cuffed murine arteries and subsequent real-time RT-PCR established in vivo induction of SM α-actin mRNA in the media of activin A–treated mice. In summary, activin A inhibits neointima formation in vitro and in vivo by preventing SMC dedifferentiation. (Circ Res. 2002;90:1128-1134.)

Key Words: cuff model ■ adenovirus ■ transforming growth factor-β ■ smooth muscle cell ■ laser-capture microdissection

Atherosclerosis, restenosis, and stenosis in venous coronary bypasses are vascular pathologies that eventually result in occlusion of the vessel lumen.1–3 Migration and proliferation of medial smooth muscle cells (SMCs) into the neointima contributes substantially to lesion formation and involves a phenotypic change of resting, contractile cells into activated, synthetic SMCs.

Activin A is a pleiotropic, transforming growth factor-β (TGF-β)–like factor that is involved in differentiation of various cell types. Its role has been well documented in embryonic mesoderm induction,4 erythroid differentiation,5 and the determination of hepatic organ mass.6 Activin A has also been identified as a factor that is expressed in the atherosclerotic vessel wall7–9 and may change SMC proliferation, although its effect is controversial; activin A has been described to enhance rat SMC DNA synthesis in some studies,8,10 whereas others reported that activin A did not affect rat SMC growth.9,11 Notably, we have shown that activin A expression is upregulated at the level of mRNA, protein mass, and bioactive protein in human atherosclerotic tissue.9 Furthermore, activin A was demonstrated to alter the characteristics of cultured SMCs toward a differentiated, contractile phenotype, associated with nonatherogenic, media-derived SMCs.9 Based on these observations, we hypothesized that activin A reduces neointima formation by preventing phenotypic modulation of vascular SMCs. This assumption may be relevant to improve the functional life span of coronary vein grafts, which at present perform unsatisfactorily with a patency of only 40% at 10 years after surgery.12 Previous research on adenovirus-mediated inhibition of neointimal hyperplasia has focused on inhibition of the altered properties of activated SMCs rather than on preventing the acquisition of these traits. Gene therapy strategies to reduce proliferation of SMCs have been performed by interfering with the cell cycle or with apoptosis. Consequently, these approaches aimed at enhancing the activity of p21,13 retinoblastoma,14 and FasL.15 Furthermore, inhibition of matrix degradation and migration has been assessed by overexpression of tissue inhibitors of metalloproteinases TIMP-1,16–17 TIMP-2,18 and TIMP-319 or protease...
inhibitors like ATF-BP1. Maintaining the contractile SMC phenotype would represent a novel strategy to prevent intimal hyperplasia. In this report, we assessed the validity of this phenotype would represent a novel strategy to prevent intimal hyperplasia. In this report, we assessed the validity of this phenotype would represent a novel strategy to prevent intimal hyperplasia. In this report, we assessed the validity of this phenotype would represent a novel strategy to prevent intimal hyperplasia. In this report, we assessed the validity of this phenotype would represent a novel strategy to prevent intimal hyperplasia. In this report, we assessed the validity of this

**Materials and Methods**

**Construction of Activin A–Expressing Adenovirus (Ad.activin)**

Human activin A cDNA was inserted into the pCMV adenoviral shuttle vector. The activin A cDNA-containing vector and plasmid pM17 were cotransfected into HER 9.11 cells, and subsequently viable clones were selected, using a standard plaque isolation, followed by amplification and purification. Similarly, adenoviral constructs carrying β-galactosidase DNA (Ad.lacZ) or no insert (Ad.mock) were generated as described. The particle per plaque-forming unit (pfu) ratio was for all adenovirus preparations between 15 and 20. Human arterial SMCs were infected with Ad.activin and Ad.mock (2×10⁸ pfu/mL), and supernatants were collected after 48 hours.

**Western Blotting**

Conditioned media from cultured cells or saphenous vein organ cultures, which were infected with Ad.activin or Ad.mock, were assayed. Purified recombinant human activin A (lot No. 15365-36) and follistatin (lot No. B3904) were obtained from Dr. Parlow through the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Disease (Bethesda, Md). The samples were analyzed by 12.5% (wt/vol) SDS-PAGE. To detect activin A protein, the E4 antibody (Serotec) was applied in Western-blotting protocols.

**Activin A Bioactivity Assay**

T47D cells were cultured in 24-wells plates (Nunc) in DMEM/F12 medium, supplemented with 5% (v/v) fetal calf serum (FCS) (Gibco). At half maximal cell density, the cells were incubated in DMEM/F12 medium without phenol-red, supplemented with 5% charcoal-tREATED FCS. After 24 hours, the cells were incubated with purified activin A (50 ng/mL) or conditioned media, and inhibition of proliferation of T47D cells was measured by 3H-thymidine incorporation. To demonstrate the specificity of activin A, control samples were incubated with 250 ng/mL purified follistatin, which is a physiological inhibitor of activin A.

**Human Saphenous Vein Organ Culture**

Segments of human saphenous veins were obtained from patients undergoing coronary bypass graft surgery, according to the guidelines of the Medical Ethical Board of the Academic Medical Center. Leftover fragments of saphenous veins were subdivided in segments of 1 cm. Longitudinally cut segments were infected for 1 hour at room temperature with 5×10⁸ pfu/mL of Ad.activin or Ad.lacZ. After 15 hours, ECs were infected with viruses (10⁹ pfu in 200 μL) and supernatants were collected after 48 hours.

**SMC Cultures and Northern Blotting**

Explant cultures of SMCs derived from human saphenous vein and mouse aorta were maintained in 40% to 40% M199-RPMI1640 medium/20%FCS, supplemented with penicillin/streptomycin (Gibco). As described, SMCs were immunohistochemically characterized and subsequently treated with activin A, total RNA was isolated, and Northern blots were manufactured and hybridized with either a SM22α cDNA probe or an oligonucleotide to detect human SM α-actin. To assess murine SM α-actin expression, the oligonucleotide 5'-TATGTGTGAAGAGGAAGACAGC-3' was applied. Saphenous vein SMCs and murine artery SMCs were either not infected or infected with Ad.activin or Ad.mock (10⁹ pfu/mL) and after 1 week the cells were fixed and immunostained for the presence of SM α-actin, applying a FITC-conjugated secondary antibody.

**Real-Time RT-PCR**

From paraffin sections (5 μm), derived from cuffed arteries after 3 weeks, the medial SMC layers were isolated by laser-capture microdissection (Leica AD LMD, Leica Microsystems). First, the lumen and intimal tissue were dissected at the internal elastic lamina and removed, and subsequently, medial tissue was taken at the external elastic lamina and harvested. Ten sections were dissected from 2 different cuffed arteries per experimental group (Ad.mock- and Ad.activin-infected mice), and the tissue was collected in an RNase-free microtube. The tissue was incubated in 10 μL with 10 U of RNAse (Promega) in 10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10 mmol/L MgCl₂ for 10 minutes at 95°C. Subsequently, an RNase-free DNase treatment (Gibco) was performed for 30 minutes at 37°C. After complete inhibition of the DNase during 10 minutes at 65°C in the presence of excess EDTA, first strand cDNA synthesis was performed with Superscript (Gibco) according to the manufacturer’s protocol. As a control, the enzyme was omitted from the reaction. In these RNA preparations, the mRNA level of SM α-actin was determined with the following oligonucleotides: 5'-TCCCTGGAGAAGAGCTACGAACT-3' and 5'-GATGCCGCTGACTCCAT-3'. As control for total mRNA content, the mRNA expression of the ribosomal housekeeping gene 36B4 (HKG 36B4) was assessed with the following oligonucleotides: 5'-GGACCCGAGAAGACCTCCTT-3' and 5'-GACATCACTCAAGATTTCAATGG-3'. Semiquantitative PCR was performed using the PCPR for SYBR Green Core kit (Eurogentec) applying an ABI PRISM 7700 sequence detection system (Perkin Elmer Biosystems).
Results

Adenovirus-Mediated Activin A Expression
To investigate both activin A protein synthesis and activin A bioactivity on adenoviral infection, primary SMCs were infected with Ad.activin. The conditioned media of Ad.activin- and Ad.mock-infected SMCs were compared with purified activin A by SDS-PAGE and Western blotting (Figure 1a). Under nonreducing conditions, a 24-kDa band is observed of dimeric activin A. After reduction of the samples, a doublet is observed of 14 kDa, representing differentially glycosylated, monomeric activin A. The higher Mr-band in the supernatant of Ad.activin-infected cells is due to incomplete processing of activin A, which occurs when high expression levels are reached. No activin A antigen was synthesized in Ad.mock-infected cells. Second, activin A bioactivity was tested using an assay based on activin A–dependent inhibition of proliferation of T47D breast-tumor cells. Indeed, we demonstrate that the conditioned medium of Ad.activin-infected SMCs reduces T47D proliferation, whereas the conditioned medium of Ad.mock-infected cells has no effect (Figure 1b). Addition of follistatin, the physiological inhibitor of activin A, relieves the inhibition exerted by activin A.

Effect of Activin A on Neointima Formation in Human Saphenous Vein Organ Cultures
The human saphenous vein organ culture is a well-defined model system in which a multilayer neointima is formed, consisting of SM α-actin positive SMCs and extracellular matrix components. Saphenous vein segments, derived

Figure 1. Characterization of activin A–expressing adenovirus (Ad.activin). a, Western blot analysis of the conditioned medium of primary SMCs, infected with Ad.mock or Ad.activin. The samples were subjected to SDS-PAGE either under reducing (R) or nonreducing (NR) conditions. Purified activin A was used as a control. Molecular weight markers (prestained) are shown in the lane marked M. b, Activin A bioactivity assay. The proliferation of human T47D breast tumor cells is inhibited by activin A as determined by measuring 3H-thymidine incorporation. Purified activin A, conditioned media of Ad.activin, or Ad.mock-infected SMCs were analyzed. Follistatin (Fol) was added to demonstrate activin A specificity. Bioactivity is expressed in arbitrary units (A.U.) as (3H-thymidine incorporation (cpm) of activin A+Fol sample)/(3H-thymidine incorporation (cpm) of tested sample).

Figure 2. Effect of activin A in human saphenous vein organ cultures. Saphenous vein segments remained untreated (Control, a), were infected with Ad.lacZ (b) or with Ad.activin (c), and were cultured for 5 weeks. Subsequently, the veins were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Photomicrographs of typical examples are shown (magnification ×25). The neointimal tissue is overlying the original surface of the vessel, which is indicated by a dotted line. Western blot analysis (d) of conditioned medium, collected after a 7-day culture period, from untreated (control), Ad.lacZ−, or Ad.activin-treated saphenous vein segments. Purified activin A is applied as a control. Molecular weight markers (prestained) are shown in the lane marked M.
from the same patient, were either not infected (Figure 2a) or infected with Ad.LacZ (5 × 10⁹ pfu/mL), resulting in a reproducible neointimal hyperplasia (Figure 2b). Adenoviral infection directs expression of genes to adventitial cells and to a lesser extent to neointimal and medial cells (see online Figure 4 in the online data supplement available at http://www.circresaha.org). Importantly, virtually no neointima formation was observed in Ad.activin-infected segments (5 × 10⁹/circresaha.org). Importantly, virtually no neointima formation was observed in Ad.activin-infected segments (5 × 10⁹/circresaha.org). Neointima formation is expressed as [(neointima area)/(media area)] × 100%. NS indicates not significant (P > 0.05); ***P < 0.001.

Figure 3. Morphometry of neointima formation in human saphenous vein culture. Morphometric analysis of surface areas of the neointima and media of independent cultured saphenous veins (n = 6). Noninfected cultures (control, white bar) infected with Ad.lacZ (gray bar) or infected with Ad.activin (black bar). Neointima formation is expressed as ([neointima area]/[media area]) × 100%. NS indicates not significant (P > 0.05); ***P < 0.001.

Effect of Ad.activin Infection on Neointima Formation In Vivo

To assess the effect of activin A in vivo, a SMC-rich neointima was provoked in FVB mice by placing a 0.4-mm nonrestrictive polyethylene cuff around the left femoral artery, resulting in neointimal hyperplasia within 3 weeks.²⁰ Simultaneous to cuff placement, the mice were infected with Ad.activin (10⁹ pfu) or with Ad.mock (10⁹ pfu) as a control infection or were not infected. Systemic adenovirus infection mainly targets hepatic tissue, resulting in high levels of expression of activin A mRNA and protein in the liver, whereas in Ad.mock-treated mice neither activin A mRNA nor protein was detected (see online Figure 5). Morphometric analysis of the cuffed murine femoral arteries demonstrated that, after 3 weeks, a neointima had formed in control mice that consisted of 4 to 6 cell layers of SMCs (Figures 4a and 4b). Mice that were infected with Ad.mock developed a neointima with a similar size (Figures 4c and 4d). Most importantly, Ad.activin-treated animals showed a strongly reduced development of neointimal tissue or even complete absence of neointimal hyperplasia (Figures 4e and 4f). No significant effects were observed on the size or circumference of the media of the murine femoral arteries. Neointima/media ratios from the noninfected mice (149 ± 48%) and Ad.mock-infected mice (154 ± 67%) were similar, whereas Ad.activin (35 ± 10%) infection resulted in 77% reduction of neointima formation (Figure 5).

Effect of Activin A on Cultured SMCs and on Medial SMCs of Cuffed Murine Arteries

To further investigate the effect of activin A on human saphenous vein SMCs and mouse SMCs, we cultured these cells in the presence of purified activin A for different time periods and assessed the expression of the SM-specific genes SM22α and SM α-actin. Expression of these genes is enhanced 2- to 4-fold both in human venous SMCs and in

Figure 4. Effect of activin A on cuff-induced neointima formation in mouse femoral arteries in vivo. After cuff placement, the mice were either noninfected (Control, a and b), or infected with Ad.mock (c and d) or with Ad.activin (e and f). After 3 weeks, the mice were euthanized, and cross sections were obtained and analyzed. In the higher magnifications (bottom), the neointimal tissue, overlying the internal elastic lamina (dotted line), is shown in more detail (b, d, and f). Original magnification of the photomicrographs ×200 (a, c, and e) and ×630 (b, d, and f).
mouse arterial SMCs within 24 hours of activin A treatment (Figure 6a). In addition, human venous SMCs and mouse arterial SMCs were infected with Ad.activin, Ad.mock (10^8 pfu/mL), or were left uninfected, and the expression of SM α-actin mRNA in the media of cuffed arteries of Ad.activin-infected and Ad.activin-infected mice (Figures 6c and 6d). To avoid any bias from the dramatic difference in lesion content in lesions of the different experimental groups, we isolated only medial SMCs from sections by laser-capture microdissection (Figure 6c). Real-time RT-PCR was used to investigate the level of SM α-actin mRNA in the media of cuffed arteries of Ad.activin-treated mice compared with the SM α-actin content in media of Ad.mock-treated mice, after correction for the amount of cDNA as determined by the expression level of a housekeeping gene (HKG 36B4). Melting curve analysis and primer efficiency of the 2 amplicons were verified (data not shown). We determined ΔΔC_t as the difference of SM α-actin cycle number between Ad.activin and Ad.mock samples, after subsequent subtraction of the cycle numbers for the housekeeping gene. The regulation of SM α-actin expression was calculated as 2^−ΔΔC_t-fold induction.24 Real-time RT-PCR revealed a 4- to 8-fold increase of SM α-actin mRNA in the media of cuffed arteries of Ad.activin-treated mice, and in Figure 6d, a typical example (8-fold induction) is shown. The variation in extent of induction was dependent on the batch of microdissected tissues assayed. These results indicate that activin A induces both in vitro and in vivo the expression of SMC-specific markers.

**Discussion**

Atherosclerosis, restenosis after angioplasty, and vein graft disease involve SMC dedifferentiation toward a proliferating phenotype. Previous approaches to counteract SMC-rich neointima formation include adenoviral transfer of various genes interfering with either cell proliferation or inhibiting cell migration. In contrast, our study focused on prevention of SMC activation by retaining a differentiated, quiescent phenotype. Based on our previous research,9 we propose that activin A meets the criteria of a factor that maintains SMCs in their nonproliferative state. In line with our expectations, in the present study, we show a vast reduction (almost 80%) of neointima formation by activin A both in the saphenous vein organ cultures and in the murine cuff model. We hypothesize that the mechanism of action of activin A is based on modulation of SMC phenotype. A typical characteristic of SMCs is their flexibility in phenotype transition in response to local environmental stimuli. The most extreme phenotypes are at the one end the quiescent, contractile SMCs such as present in the media of the normal, fully distensible vessel wall and at the other end the proliferating, migrating SMCs that synthesizes excessive amounts of extracellular matrix compounds and is present in developing (restenotic) lesions. It is conceivable that SMCs exhibit a spectrum of intermediate phenotypes and may switch back and forth in this range. We observed that activin A does not affect DNA synthesis of cultured SMCs, as determined by 3H-thymidine incorporation (online Figure 1). In this respect, our results are in agreement with data published by Nakaoka et al.11 Furthermore, we evaluated the effect of activin A on migration of cultured SMCs in a trans-well migration assay and concluded that activin A does not affect (platelet-derived growth factor–mediated) SMC migration (online Figure 2). Finally, we assayed extensively, using different test systems (Annexin V staining with propidium iodine exclusion, TUNEL) for changes in apoptosis in cultured SMCs and in sections of cuffed arteries and observed no differences in programmed cell death in response to activin A (online Figures 3 and 6). However, we convincingly showed that activin A enhances in cultured human saphenous vein SMCs, in cultured murine arterial SMCs, and in the media of cuffed arteries the expression of the SM-marker SM α-actin.

Our data may therefore indicate that activin A affects the phenotype of SMCs toward the quiescent state. Although no immediate effects on proliferation and migration are observed in cultured SMCs, prolonged exposure of SMCs to activin A, which is realized in the organ cultures and murine experiments shown in this study, may prevent full activation of medial SMCs.

The experimental models applied in this study focus on the role of SMCs in lesion formation and do not allow extensive conclusions on the effect of activin A on endothelial cells and macrophages in this process. In the saphenous vein organ culture, limited numbers of endothelial cells are present, whereas no macrophages are involved. In the cuff model, eventually a SMC-rich lesion is formed, but both endothelial cells and macrophages are involved in the initiation of lesion formation and consequently a potential effect of activin on these cell types cannot be excluded. We aim to delineate the exact effect of activin A on these cell types in models in which more complex macrophage-rich lesions are being formed.

Bone morphogenetic protein-2 (BMP-2), another member of the TGF-β superfamily, has been demonstrated to reduce neointima formation in response to balloon injury of the rat carotid artery.11 Similar to the effect observed for activin A,
no matrix deposition was observed in the vessel wall. However, application of BMP-2 yielded inhibition of neointimal hyperplasia of only 41%. These data on BMP-2 and activin A are in contrast to results obtained on delivery of the TGF-β/H9252 gene or protein to uninjured porcine and rat arteries, and to injured rabbit arteries. In each of those models, TGF-β/H9252 induces excessive neointimal extracellular matrix deposition.25–27 In the rabbit injury model, the 2-fold enhancement of lesion formation did not coincide with an increased number of cells in the intima, whereas the other studies describe TGF-β/H9252–dependent cellular proliferation. These differences in vascular response between activin A, BMP-2, and TGF-β are not explained by the presently available knowledge on downstream Smad-signaling. TGF-β/H9252 and activin A both induce the phosphorylation and subsequent nuclear translocation of Smad2 and Smad3 on binding and activation of different, growth factor–specific Type I and Type II serine/threonine-kinase receptors. In contrast, activation of BMP receptors results in phosphorylation of Smad1, 5, and 8.28 Recently, it has been shown that TGF-β induces fibronectin synthesis through a Smad-independent pathway involving c-Jun N-terminal kinase.29 So far, this cascade has not been identified to contribute to activin-dependent signal transduction, which may explain the lack of neointimal extracellular matrix production in mice in response to activin A.

We assume that activin A reduces neointima formation directly, although no conclusive evidence can be provided. This assumption is based on the knowledge that the effect of activin A is apparently independent of its site of synthesis. In the saphenous vein-cultures, activin A is synthesized on adenoviral infection of vascular cells, whereas in the mouse experiments, activin A is programmed by the adenovirus and made in the liver and still comparable inhibition of hyperplasia has been observed in both model systems.

In conclusion, we demonstrate that activin A inhibits the formation of SMC-rich hyperplasia, and we propose that activin A is a protective factor that maintains a more contractile SMC phenotype.

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Figure 1:
DNA synthesis in SMC and HUVEC in response to activin A and TGFβ as determined by $^{3}$H-thymidine incorporation.

Human umbilical cord artery smooth muscle cells (SMC) (a) and human umbilical cord vein endothelial cells (HUVEC) (b) were incubated with increasing concentrations of activin A (circles) or TGFβ (inverted triangles) in serum-free medium (open symbols) or in medium containing 10% serum (closed symbols). $^{3}$H-thymidine incorporation is expressed as the percentage of counts incorporated in cells treated with growth factors over the counts incorporated in cells grown in serum.

Activin A does not affect DNA synthesis in SMC and inhibits the growth of endothelial cells. Under the culture conditions applied TGFβ inhibits DNA synthesis in SMC.
Figure 2.
Migration of human saphenous vein SMC in response to activin A and PDGF in transwells.

The migration assay was performed as described previously (Lamfers et al, Thromb Haemost 2000;84:460-67). 4x10⁴ cells were seeded in the upper chamber of the transwells and cultured for 20 h. The upper chamber was replaced with DMEM (supplemented with 0.15 μM plasminogen in the matrigel-coated well) and in the lower chamber 10% FCS was added as chemoattractant with additionally activin A (100 ng/ml) and/or platelet derived growth factor (PDGF; 10 ng/ml). After 4 days the cells were fixed. Cells on the upper surface were scraped off, and cells on the lower side of the filter were stained with crystal violet. Migration was determined as the % surface covered by cells by image analysis. Data were analyzed using Optimas image analysis software (Bioscan Inc).

Activin A does not affect SMC migration in the absence or in the presence of PDGF.
Figure 3

**Figure 3.**
**Apoptosis of SMC in response to activin A as determined by Annexin V binding.**
Human saphenous vein SMC were grown to confluency and kept O/N. The cells were treated for the indicated times with staurosporine (0.25 µM), activin A (100 ng/ml) or TGFβ (10 ng/ml). Subsequently, the cells were trypsinized and incubated with Annexin V-FITC and propidium iodide and kept on ice. By FACS analysis the relative number of cells that were Annexin V-positive, excluding PI-positive cells, was determined as a measure for apoptosis. **Activin A does not induce apoptosis in cultured SMC.**
Figure 4.
Expression of β-Galactosidase in saphenous vein organ cultures treated with Ad.lacZ.
Segments of saphenous veins were either infected with Ad.lacZ and placed in culture for 5 weeks. The veins were analyzed whole mount for β-Galactosidase activity and either photographed immediately, or embedded in paraffin and sectioned. β-Galactosidase activity results in a blue precipitate after X-gal staining. En face view from the adventitial side (a) or from the intimal side (b). Photomicrograph of section after X-gal staining (c) Original magnifications 10x (a, b) and 25x (c)
Adenoviral infection directs the expression of genes to adventitial cells and to a lesser extent to neointimal and medial cells.
Figure 5.
Activin A expression in the liver after systemic infection of mice.
Photomicrographs of cross-sections of murine livers, obtained from Ad.mock (a, c) and Ad.activin (b, d) infected mice are shown. Activin A mRNA expression was detected by radioactive in situ hybridization and visualized by epipolarization (bright-blue dots on a blue background; a, b). Activin A protein expression was revealed with immunohistochemistry applying a specific antibody (E4, Serotec) and shows a brick-red positive staining on a blue hematoxylin counterstaining (c, d). Original magnification of the photomicrographs 200x. Activin A mRNA and protein is expressed in the liver of Ad.activin infected mice.
Figure 6

Figure 7.
Detection of apoptotic cells in murine femoral artery after cuff placement in the presence of control virus or Ad.activin.

After cuff placement, the mice were either infected with Ad.mock (Control; a, c, d), or infected with Ad.activin (b, e). After three weeks the mice were sacrificed and cross sections were obtained and analyzed by the TUNEL technique (a-c) (Kockx et al, Circulation 1998;97:2307-15.) or by immunohistochemistry applying an antibody directed against cleaved (=activated) Caspase-3 (d-f) (polyclonal antibody D175, Cell Signaling, Beverly, MA). No apoptotic cells were observed in media or neointima of cuffed arteries in mice treated without (a, c, d) or with activin A (b, e). TUNEL-positive cells are present in the granulation tissue surrounding the cuff, independent of the treatment (c). As a control for specificity of the Caspase-3 antibody, apoptotic cells were identified in normal mouse colon (f). In d and e the internal elastic lamina is highlighted with a line). Original magnifications 100x (a, b), 50x (c), 200x (d-f).

Activin A does not induce apoptosis in cuffed, murine femoral arteries.