Expression of Follistatin, an Activin-Binding Protein, in Vascular Smooth Muscle Cells and Arteriosclerotic Lesions

Satoshi Inoue, Akira Orimo, Takayuki Hosoi, Takeshi Matsuse, Makoto Hashimoto, Ryutaro Yamada, Yasuyoshi Ouchi, Hajime Orimo, Masami Muramatsu

Activin-A has a mitogenic effect on vascular smooth muscle cells (SMCs) and is produced by monocyte/macrophage lineage cells. Here, we studied the expression of follistatin, an activin-binding protein, in both A10 cells, a rat aortic SMC line, and vascular SMCs derived from adult rat aorta. Follistatin mRNA was detected in these cells by Northern blot analysis. Ligand blot and immunoblot analyses demonstrated that follistatin was produced in the conditioned medium at a higher level by A10 cells than by SMCs. Furthermore, immunostaining and in situ hybridization of the arteriosclerotic lesions showed that follistatin was highly expressed in the diseased artery, where abnormal proliferation of SMCs occurred. We suggest that follistatin is produced by vascular SMCs and is involved in the course of atherogenesis. (Arterioscler Thromb. 1993;13:1859-1864.)

KEY WORDS • follistatin • activin • arteriosclerosis • smooth muscle cells

Smooth muscle cell (SMC) proliferation in vivo plays a central role in the pathogenesis of atherosclerosis.1,2 Atherogenic stimulations induce dedifferentiation, abnormal proliferation, and migration of the vascular SMCs, resulting in formation of neointimas where monocyte-derived macrophages are abundant.3 Involvement of various cytokines and monokines has been reported in the proliferation and differentiation processes of SMCs. These include transforming growth factor (TGF)-β, which is involved in divergent aspects of cell differentiation.4 Vascular SMCs have TGF-β receptors5 and also produce TGF-β1,6 which can proliferate vascular SMCs by an autocrine mechanism and affect their migration.7 It has been reported8,9 that the expression of TGF-β1 mRNA in rat aortic tissues is increased in experimental hypertensive rats compared with normotensive rats, suggesting some roles of TGF-β1 in mediating the aortic changes induced by hypertension. Ross et al.10 report that transcripts for platelet-derived growth factor B (PDGF-B), c-fms, interleukin-1, and TGF-β are increased in the advanced lesions of atherosclerosis induced in nonhuman primates maintained on a hypercholesterolemic diet for 1 year. They suggested a significant role of cytokines, including PDGF-B produced by macrophages, on atherosclerosis.

Activin was originally isolated from ovarian fluids as a stimulator of follicle-stimulating hormone (FSH) secretion from the pituitary gland.11 This molecule belongs to the TGF-β family, but recent analyses show that it as well as TGF-β1,12 have diverse biological roles besides causing hormonal effects on the reproductive system. Activin acts as an erythroid differentiation factor,13 an inhibitor of neural differentiation14 or a survival factor of neural cells,15 and as a mesoderm-inducing factor in early amphibian development.16-18 Follistatin, which was isolated as an inhibitor of FSH secretion from the pituitary gland,19 was found to bind with activin and inhibit its activity.20,21 Follistatin as well as activin is presumed to have a significant role in extraglandular tissues.22-25 We have reported that neural cells produce follistatin to interact with exogenously derived activin26 and that the function of MC3T3-E1 cells, an osteoblastic cell line, is regulated by activin and follistatin.27 However, little is known about the role of activin and follistatin in vascular tissues. Kojima et al.28 report that activin-A has a mitogenic effect on vascular SMCs from rat aorta and that this effect of activin-A is different from that of TGF-β1. Activin-A increased the number of vascular SMCs after 30 hours of incubation. Activin-A is produced by monocyte/macrophage lineage cells,13 which are abundant in arteriosclerotic lesions.3 We suspected that a mitogenic effect of activin-A on vascular SMCs might be involved in neointimal proliferation. Moreover, because activin and follistatin are involved in the morphogenesis of early development,16-18 we suspected that they might also be involved in the morphogenesis or regeneration that occurs in arteriosclerotic lesions.

In this article we report on an investigation of the expression of follistatin in vascular SMCs and in arteriosclerotic lesions. The expression of follistatin both at the mRNA level and at the protein level was demonstrated in vascular SMCs. Furthermore, follistatin was...
highly expressed in the affected arteries, suggesting some roles of follistatin in arteriosclerosis.

Methods

Cell Culture
A rat aortic SMC line, A10 cells, was purchased from the American Type Culture Collection. A10 cells were maintained in an alpha modification of Eagle's minimum essential medium (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratories) at 37°C in a humidified atmosphere of 5% CO₂. SMCs were cultured from the thoracic aorta of 8-week-old Wistar rats by the method of Chamley et al. Primary cultures of SMCs were established from the medial layer of the aorta and were subcultured in α-MEM supplemented with 10% FBS.

Materials
Recombinant activin-A, produced by using Chinese hamster ovary cells, was a kind gift from Drs Y. Eto and H. Shibai, Central Research Laboratories, Ajinomoto Co, Kawasaki, Japan. Porcine follistatin and rabbit anti-human follistatin polyclonal antibody were a generous gift from Dr H. Sugino, The Institute for Enzyme Research, Tokushima University, Tokushima, Japan. Experimental animals were purchased from Saitama Animal Laboratory, Saitama, Japan.

Northern Blot Analysis of A10 Cells and SMCs

Probed With Mouse Follistatin

Northern blot analysis of A10 cells and SMCs was performed as described with minor modifications. A10 cells (10⁵/mL) and SMCs (10⁵/mL) were incubated in culture dishes for 2 days. The conditioned medium (20 mL) was collected, combined with 1 g sulfate-Cellulofine (Seikagaku Kogyo, Tokyo, Japan), and stirred gently for 12 hours at 4°C. The gels were washed twice with 4 mL of 0.35 mol/L NaCl and 20 mmol/L Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, washed once with distilled water, and eluted with 1% SDS and 100 mmol/L Tris-HCl, pH 7.5. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (12%) and were electroblotted on Immobilon membranes (Millipore) by the semidyve transfer system. As a negative control, 20 mL α-MEM containing 10% FBS was also treated in the same manner. Follistatin 500 ng purified from porcine ovaries was blotted as a positive control. The blots were then washed with TBS, incubated in the same manner. Follistatin 500 ng purified from porcine ovaries was blotted as a positive control. The blots were then washed with TBS, incubated with affinity-purified goat antibody to rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Sigma) at a 1:1000 dilution in 1% skim milk in TBS for 2 hours at room temperature, and finally washed with 0.1% Triton X-100 in TBS for 30 minutes. The proteins were developed with 4-chloro-1-naphthol. Ligand blots were incubated in 10% skim milk overnight and treated with 100 ng/mL ¹²⁵I-activin-A in TBS containing 0.1% Triton X-100 for 2 hours at room temperature. They were then washed with TBS containing 0.1% Triton X-100 for 1 hour, and the autoradiograph was taken by 4 days' exposure at -80°C with an intensifying screen. Three repetitions were performed for each experiment, and a representative pattern is shown.

Immunoblot and Ligand Blot Analysis of Follistatin in the Conditioned Medium of A10 Cells and SMCs

Immunoblot and ligand blot analyses were done as described with minor modifications. A10 cells (10⁵/mL) and SMCs (10⁵/mL) were incubated in culture dishes for 2 days. The conditioned medium (20 mL) was collected, combined with 1 g sulfate-Cellulofine (Seikagaku Kogyo, Tokyo, Japan), and stirred gently for 12 hours at 4°C. The gels were washed twice with 4 mL of 0.35 mol/L NaCl and 20 mmol/L Tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, washed once with distilled water, and eluted with 1% SDS and 100 mmol/L Tris-HCl, pH 7.5. The proteins were resolved by SDS-polyacrylamide gel electrophoresis (12%) and were electroblotted on Immobilon membranes (Millipore) by the semidyve transfer system. As a negative control, 20 mL α-MEM containing 10% FBS was also treated in the same manner. Follistatin 500 ng purified from porcine ovaries was blotted as a positive control. For immunoblotting, the blots were first blocked for 1 hour with 5% skim milk in Tris-buffered saline (TBS) and then incubated with rabbit anti-human follistatin polyclonal antibody at a 1:500 dilution for 2 hours at room temperature. The blots were then washed with TBS, incubated with affinity-purified goat antibody to rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Sigma) at a 1:1000 dilution in 1% skim milk in TBS for 2 hours at room temperature, and finally washed with 0.1% Triton X-100 in TBS for 30 minutes. The proteins were developed with 4-chloro-1-naphthol. Ligand blots were incubated in 10% skim milk overnight and treated with 100 ng/mL ¹²⁵I-activin-A in TBS containing 0.1% Triton X-100 for 2 hours at room temperature. They were then washed with TBS containing 0.1% Triton X-100 for 1 hour, and the autoradiograph was taken by 4 days' exposure at -80°C with an intensifying screen. Three repetitions were performed for each experiment, and a representative pattern is shown.

Experimental Arteriosclerosis With Intimal Denudation

The left common carotid arteries of four male 12-week-old Sprague-Dawley rats were mechanically injured by passing a balloon catheter along the vessel several times. After 2 weeks the animals were killed, and the arteries with a thickened neointima and those without arteriosclerosis on the other side were excised and used for immunostaining.

Immunostaining

The tissues obtained above were fixed, dehydrated, and embedded in paraffin wax. Two-micrometer sections were mounted on coated glass slides and subjected to immunostaining. Briefly, after being deparaffinized, sections were soaked in methanol containing 0.03% H₂O₂ to block endogenous peroxidase and were then treated with 10% normal goat serum in phosphate-buffered saline (PBS) for 30 minutes. The sections were incubated overnight at 4°C with the rabbit polyclonal antibody against human follistatin diluted 1:500 with 10% PBS-PBS. The antibody was confirmed to be suitable for immunostaining of rat tissues. Anti-muscle actin antibody HHHF35 (Enzo Diagnostics, New York, NY) was used for smooth muscle detection, and normal rabbit serum was used as a negative control. After the incubation, the sections were incubated with goat anti-rabbit IgG antibody conjugated with biotin (Nichirei, Tokyo, Japan) for 1 hour at room temperature, washed with PBS, and then treated with streptavidin conjugated with horseradish peroxidase (Nichirei). For the staining with HHHF35, rabbit anti-mouse IgG antibody conjugated with biotin (Nichirei) was used as a second antibody. Specifically bound antibodies were visualized by immersing the sections in a substrate solution of 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, Calif). Counterstaining of nuclei was done with 1% Mayer's hematoxylin solution. The tissues were examined under a light microscope.

Experimental Arteriosclerosis With a High-Cholesterol Diet

Four 16-week-old male New Zealand White rabbits with normal serum cholesterol levels were maintained on a diet containing 2% (wt/body wt) cholesterol. Four
Follistatin Expression in Arteriosclerosis

Inoue et al

**Northern blot analysis showing detection of follistatin mRNA.** Poly(A)+ RNA was extracted from A10 cells (3 μg each) without retinoic acid (RA−; lane 1) or with 1 μmol/L RA (RA+; lane 2); from 15 μg smooth muscle cells (SMCs; lane 3); and from 3 μg rat uterus (lane 4) and was probed with a mouse follistatin or β-actin cDNA probe.

Rabbits were maintained on a normal diet as a negative control. Rabbits were killed after 20 weeks, and the aorta with atheromatous plaques was excised and prepared for in situ hybridization. Aorta and testis were obtained from normal-diet rabbits and were processed in an identical manner.

**In Situ Hybridization**

Tissues from rabbit aorta and testis were fixed in 4% phosphate-buffered paraformaldehyde and embedded in paraffin wax. Four-micrometer sections were mounted on coated glass slides and subjected to in situ hybridization. Briefly, after being deparaffinized, sections were treated with 0.2N HCl for 10 minutes, digested with 50 μg/mL RNase-free proteinase K for 15 minutes, and fixed with 4% paraformaldehyde, followed by acetylation. The sections were incubated with 20 μL of a hybridization mixture containing 15 ng of a digoxigenine-11-dUTP-labeled probe for follistatin at 37°C overnight. The cDNA probe for follistatin was a 622-bp fragment that was labeled with digoxigenine-11-dUTP by using a random-primed DNA labeling kit (Boehringer, Mannheim, FRG). After washing off the unbound probe, the sections were incubated with alkaline phosphatase–conjugated anti-digoxigenine antibody. p-Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphosphate p-toluidine salt were used to visualize the site of hybridization. As a negative control, an RNase-treated section from each block was processed in the same manner.

**Results**

**Expression of Follistatin mRNA in Both A10 Cells and SMCs**

The expression of follistatin mRNA (Fig 1) was detected in both A10 cells and SMCs by Northern blot analysis probed with a mouse follistatin cDNA fragment. The two bands that were detectable corresponded to the bands observed in rat uterus as the positive control. The amounts of follistatin mRNA in SMCs and A10 cells were less than that in uterus but yet significant. When A10 cells were treated with RA under 0.3% FBS for 48 hours, the expression of follistatin was decreased (Fig 1, lanes 1 and 2).

**In Situ Hybridization**

Tissues from rabbit aorta and testis were fixed in 4% phosphate-buffered paraformaldehyde and embedded in paraffin wax. Four-micrometer sections were mounted on coated glass slides and subjected to in situ hybridization. Briefly, after being deparaffinized, sections were treated with 0.2N HCl for 10 minutes, digested with 50 μg/mL RNase-free proteinase K for 15 minutes, and fixed with 4% paraformaldehyde, followed by acetylation. The sections were incubated with 20 μL of a hybridization mixture containing 15 ng of a digoxigenine-11-dUTP-labeled probe for follistatin at 37°C overnight. The cDNA probe for follistatin was a 622-bp fragment labeled with digoxigenine-11-dUTP by using a random-primed DNA labeling kit. After washing off the unbound probe, the sections were incubated with alkaline phosphatase–conjugated anti-digoxigenine antibody. p-Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphosphate p-toluidine salt were used to visualize the site of hybridization. As a negative control, an RNase-treated section from each block was processed in the same manner.

**Results**

**Expression of Follistatin mRNA in Both A10 Cells and SMCs**

The expression of follistatin mRNA (Fig 1) was detected in both A10 cells and SMCs by Northern blot analysis probed with a mouse follistatin cDNA fragment. The two bands that were detectable corresponded to the bands observed in rat uterus as the positive control. The amounts of follistatin mRNA in SMCs and A10 cells were less than that in uterus but yet significant. When A10 cells were treated with RA under 0.3% FBS for 48 hours, the expression of follistatin was decreased (Fig 1, lanes 1 and 2).
Production of Follistatin in the Conditioned Medium of A10 Cells and SMCs

Ligand blot analysis with 125I-activin-A showed the existence of follistatin-like molecules in the medium of A10 cells and SMCs (Fig 2, left). The bands derived from A10 cells (lane 3) almost corresponded to those of porcine follistatin (lane 1). The bands derived from SMCs (lane 4) were similar, though at a lower intensity than those derived from A10 cells. No binding was observed with α-medium containing 10% FBS alone (lane 2). Immunoblotting using an anti-follistatin polyclonal antibody revealed the existence of immunoreactive molecules in the medium of A10 cells (Fig 2, right, lane 7). Two forms of follistatin (32 and 35 kDa) are known to be produced by alternative splicing, and they are further modified by glycosylation. The multiple
bands appear to correspond to those molecules. The bands derived from SMCs (lane 8) were also detected but at a lower intensity than those derived from A10 cells.

Expression of Follistatin in Arteriosclerotic Lesions

Having demonstrated the capability of the SMCs to synthesize follistatin, we next investigated the expression of follistatin in experimental arteriosclerotic lesions. The left common carotid arteries of 12-week-old Sprague-Dawley rats were mechanically injured by balloon catheter. Two weeks after this procedure, the diseased arteries with thickened neointima were immunostained with anti-follistatin antibody. Fig 3a demonstrates that the neointimal and medial cells of arteriosclerotic arteries were strongly reactive to anti-follistatin antibody but not to normal rabbit serum (Fig 3b). The medial SMCs of normal arteries were weakly reactive to anti-follistatin antibody (Fig 3d). The distribution of SMCs was examined by anti-muscle actin antibody HHF35. Fig 3c shows that medial cells and most of the intimal cells were reactive to anti-muscle actin antibody. This result indicated that at least some of the immunoreactivity to anti-follistatin antibody of the medial and intimal cells of diseased arteries may be due to the SMCs in the artery.

To examine the arteriosclerotic lesions of another type of experimental model, we used rabbits fed a high-cholesterol diet. Because the anti-follistatin antibody that we used was derived from rabbits, we examined the expression of follistatin in these animals at the mRNA level. In situ hybridization (Fig 4a) shows that the follistatin mRNA was also abundant in these rabbits. Arteriosclerotic lesions, especially in the neointima. The expression of follistatin mRNA was not significant in arteriosclerotic lesions. Localized production of cyto-
kines that have mitogenic or chemotactic effects on SMCs produced by macrophages may be involved in the atherogenic process. Alternatively, activin that is detectable in human plasma may affect vascular cells via a hormonal mechanism.

On the other hand, most of the target cells of activin produce follistatin to interact with activin. Both MC3T3-E1 and P19 cells produce follistatin at the mRNA and protein levels. Furthermore, the expression of follistatin is developmentally regulated at a specific stage of differentiation, especially when cells are in rapid proliferation. It has also been shown that follistatin is expressed in various organs and tissues, such as kidney and brain, by in situ hybridization, or Northern blotting. Suggesting the involvement of follistatin in the regulation of activin-A action in extravascular tissues. These observations prompted us to study the production of follistatin in the vascular system and the involvement of follistatin in arteriosclerosis.

First, the production of follistatin mRNA was detected by Northern blotting. The results suggest that a relatively large amount of follistatin mRNA is transcribed in A10 cells (Fig 1) but that a smaller amount of follistatin is transcribed in SMCs. We then partially purified the conditioned medium of A10 cells and SMCs with sulfated cellulose gels and demonstrated the existence of follistatin-like molecules by ligand blotting or immunoblotting. These results indicate that follistatin is also produced as protein in both types of cell. The amount of protein that was produced and secreted in the conditioned medium was lower in SMCs than in A10 cells. A10 cells derived from neonatal rat tissues grow more rapidly in culture dishes than SMCs derived from primary cultures of adult rat tissues. The expression of follistatin in A10 cells was also found to be decreased when these cells were treated with RA under 0.3% FBS. Under these conditions A10 cells grow more slowly than without RA under 10% FBS (S. Inoue, June 1993, unpublished observations). These observations are in line with the expression pattern of follistatin that is developmentally and stage-specifically regulated.

Next, the expression of follistatin during the course of arteriosclerosis was investigated. In arteriosclerosis the dedifferentiation, proliferation, and migration of vascular SMCs play important roles. The SMCs in arteriosclerotic neointima are supposed to be dedifferentiated toward an embryonic phenotype, eg, by expressing a myosin heavy chain isofrom of embryonic SMCs. Some of the medial SMCs in arteriosclerosis also show an embryonic phenotype. The present work has demonstrated a high level of expression of follistatin in the arteriosclerotic arteries, where abnormal dedifferentiation and proliferation of vascular SMCs occur. The expression of follistatin in the healthy artery is detectable but at a lower degree than in the diseased artery.

We assume that RA treatment under 0.3% FBS inhibits follistatin expression in A10 cells via inhibition of growth and promotion of differentiation. Follistatin expression is higher in A10 cells than in SMCs, probably as a result of more rapid growth of A10 cells than SMCs. These in vitro observations are in line with the in vivo observations that follistatin expression is higher in arteriosclerotic lesions than in normal vessels. The finding that follistatin is highly expressed during states of rapid proliferation and dedifferentiation in SMCs could be used as a marker for dedifferentiation in the atherosclerotic process.

Follistatin in the arteriosclerotic lesion may bind with activin and inhibits its activity, namely, its mitogenic
effect on vascular SMCs.\textsuperscript{28} Alternatively, follistatin may be a carrier protein for activin because the binding affinity of activin to follistatin is weaker than that of the activin receptor.\textsuperscript{20} It is also reported\textsuperscript{26} that follistatin could bind to heparin, an antithromogenic agent, and associate with proteoglycan on the cell surface,\textsuperscript{37} the meaning of which remains to be clarified. Further investigations are required to understand the exact roles of activin, follistatin, and the complex of activin and follistatin in the course of atherogenesis.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, and Culture, Japan, and from the Foundation for Promotion of Cancer Research, Japan. We thank Dr H. Sugino for the generous gift of porcine follistatin and anti-follistatin antibodies; Dr R. Tabata for providing arteriosclerotic lesions from rabbits fed a high-cholesterol diet; and Ms M. Nomura, Ms M. H. Yamaguchi, and Ms M. Goto for technical assistance. We also thank Drs S. Nomura, I. Kojima, M. Akishita, and K. Kozaki for their helpful discussion.

References

Expression of follistatin, an activin-binding protein, in vascular smooth muscle cells and arteriosclerotic lesions.

S Inoue, A Orimo, T Hosoi, T Matsuse, M Hashimoto, R Yamada, Y Ouchi, H Orimo and M Muramatsu

doi: 10.1161/01.ATV.13.12.1859

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/12/1859

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/