Activin A as a Critical Mediator of Capillary Formation: Interaction with the Fibroblast Growth Factor Action

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Abstract. The present study was conducted to elucidate the role of activin A in capillary formation. When bovine aortic endothelial cells (BAEC) were cultured in a collagen gel, basic fibroblast growth factor (FGF-2) induced tube formation. Activin A also induced tube formation and the addition of two factors together was more effective. BAEC produced both FGF-2 and activin A as autocrine factors. Exogenous FGF-2 did not affect the production of activin A but instead upregulated the type II activin receptor. On the other hand, activin A increased the expression of FGF-2 as well as the FGF receptor. Most importantly, when the action of endogenous activin A was blocked by adding follistatin, the tubulogenic action of FGF-2 was nearly completely inhibited. Activin-induced tubulogenesis was markedly inhibited by overexpression of Smad7, an inhibitory Smad. Similarly, an inhibitor of p44/42 mitogen-activated protein (MAP) kinase attenuated the activin-mediated tubulogenesis, whereas an inhibitor of p38 MAP kinase had no effect. These results indicate that FGF-2 and activin A enhance their signals each other in BAEC, and endogenous activin A is critical for FGF-2-induced capillary formation.

Key words: Activin A, FGF-2, Tubulogenesis, Angiogenesis

ACTIVIN A, a member of the transforming growth factor-β (TGF-β), elicits various effects in diverse biological systems. These include morphogenesis and organogenesis during development, modulation of growth and differentiation of various types of cells, regulation of tissue repair and modulation of reproduction [1, 2]. Specifically, activin A also acts as an autocrine or paracrine factor to regulate branching tubulogenesis in organs such as pancreas, kidneys, and lung [3, 4]. In the vascular system, members of the TGF-β superfamily play a vital role in the regulation of vasculogenesis, angiogenesis and vascular remodeling [5]. Among the members of the TGF-β superfamily, activin A is thought to be a factor modulating growth and differentiation of endothelial cells in vitro, and angiogenesis in vivo [6–8]. Activin A also modulates growth and differentiation of smooth muscle cells in vivo and in vitro [9–11].

We previously reported that activin A plays a crucial role in tubulogenesis of endothelial cells [6]: it is synthesized in bovine aortic endothelial cells (BAEC), and the production of activin A is upregulated in response to vascular endothelial growth factor (VEGF); activin A in turn upregulates the production of VEGF and also the expression of the VEGF receptors, Flt-1 and Flk-1. Activin A and VEGF thus provide a complex autocrine loop in endothelial cells. Most importantly, the action of VEGF is nearly completely inhibited when the action of activin A is blocked [6]. These observations suggest that activin A is a critical mediator of the VEGF-induced angiogenesis. In accordance with these observations, Poulaki et al. [7] reported recently that activin A stimulates inflammatory corneal angiogenesis by increasing VEGF levels through a mitogen-activated
protein (MAP) kinase-dependent mechanism.

Basic fibroblast growth factor (FGF-2) is a multifunctional growth factor belonging to the FGF family. In addition to its growth promoting activity in various types of cells, FGF-2 is also known to be a potent regulator of angiogenesis [12]. Thus, FGF-2 induces tube formation of endothelial cells in vitro [13], and consistent with this notion, FGF-2 also promotes angiogenesis in various types of vessels in vivo [12, 14]. Accordingly, angiogenic action of FGF-2 is thought to be useful in promoting neovascularization in various ischemic diseases [15].

Interaction between the actions of activin A and FGF2 has been reported in various tissues. For example, Bao et al. reported that activin A and FGF-2 synergistically act and promote the development of neurons [16]. In the field of vascular biology, however, even though both FGF-2 and activin A are potent angiogenic factors, their interaction has not been documented. Because a mixture of growth factors is recently thought to be important for the therapeutic angiogenesis [17], we investigated the interaction of activin A and FGF-2 in tubulogenesis of endothelial cells.

Materials and Methods

Materials

Recombinant human activin A and follistatin were provided by Dr. Y. Eto of the Central Research Laboratory, Ajinomoto Inc. (Kawasaki, Japan). Recombinant human FGF-2 was provided by Kaken Pharmaceutical (Tokyo, Japan). Polyclonal anti-bovine FGF-2 was purchased from R&D Systems (Minneapolis, MN, Japan). Monoclonal anti-bovine FGF receptor-1 (FGFR1) was purchased from Zymed Laboratories (San Francisco, CA, USA). Polyclonal anti-human Smad7 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD98059 and SB202190 were purchased from Cell Signaling Technology (Beverly, MA, USA). Adenovirus vector encoding Smad7 (AdexSmad7) was provided by Dr. Hiroshi Yasuda of Showa University School of Medicine (Yokohama, Japan).

Cell culture

BAEC were purchased from Dainippon Pharamaceutical Co. Ltd. (Osaka, Japan). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA), containing 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, USA). BAEC were used from passages 6 to 16.

Measurement of tubulogenesis

BAEC were suspended at 1 × 10^5 cell/ml in a neutralized collagen solution (Koken, Tokyo, Japan), dispensed into 24-well plates, incubated at 37°C for 45 min to allow polymerization, and complete medium (DMEM containing 10% FBS) alone, or complete medium containing each cytokine was added. Photographs of the cells were taken at the indicated time using a Nikon Diaphot TMD inverted microscope (Tokyo, Japan).

To semiquantify tubulogenic activity, morphological changes after five days of culture with indicated factors were observed. Fifty colonies per experimental condition were randomly selected. Then the tube length (long axis) was measured by using image analysis software (NIH Image) [6].

Immunoblot analysis

Cells were washed three times with cold phosphate-buffered saline (PBS), suspended in Laemmli buffer, and heated to 100°C for 10 min. After centrifugation, the supernatant was collected, and the protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of protein from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinilidene difluoride membrane (Nihon Millipore, Yonezawa, Japan) by electroblotting. The membrane was treated with 5% bovine serum albumin (BSA) dissolved in Tris-saline for 1 hour at 37°C, then incubated overnight with primary antibody and washed with Tris-PBS. After incubation with horseradish peroxidase (HRP)-labeled secondary antibody for 1 hour at room temperature, the membrane was washed with Tris-PBS and analyzed using ECL Western blotting detection reagent (Amersham Life Science, Buckinghamshire, UK) and LAS3000 [6].
**RT-PCR and SYBR Green PCR**

Total RNA was isolated using the Trizol Reagent (Invitrogen Life Technologies) from BAEC. First-strand cDNA was made from total RNA using a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics., Indianapolis, USA). SYBR Green PCR was performed using the primers (Greiner bio-one Co., Tokyo, Japan) designed by the Primer Express software (Applied Biosystems Japan, Tokyo, Japan) based on published cDNA sequences for bovine FGF-2 and GAPDH. Primers used included the following: bovine FGF-2 (sense and anti-sense were 5’-GGTGTTGCTGT TGCCGAATACT-3’ and 5’-GGAGATACAGCAGT TCGAGAAGTTT-3’, respectively) and bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense and anti-sense were 5’-GCATCGTGGAGGGACT TATGA-3’ and 5’-GGGCCATCCACAGTCTTCTG-3’, respectively). The mRNA levels detected by SYBR Green analysis were normalized to GAPDH mRNA levels. SYBR Green PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems Japan) with an ABI Prism 7700 Sequence Detection System (Applied Biosystems Japan). Reactions included 25 µl of 2 × SYBR Green Master Mix, 1 µl forward primer, 1 µl reverse primer, 23 µl water and 1 µl cDNA. Samples were incubated at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, and 60 sec at 60°C.

**Quantification of FGF-2 secreted into the medium**

BAEC were cultured in 100 mm dishes with DMEM containing 10% FBS. Before the stimulation with 4 nM activin A, BAECs were incubated with serum-free DMEM for 12 hrs. For quantitative determination of FGF-2 in media, the Quantikine HS Human FGF Basic Immunoassay kit (R&D Systems) was used.

**Results**

**Effects of activin A and FGF-2 on tubulogenesis of BAEC**

As we reported previously, activin A induced tube formation in BAEC cultured in collagen gel. Compared with complete medium (Fig. 1A-a), addition of activin A promoted tube formation of BAEC (Fig. 1A-b). Quantitative analysis revealed that tubulogenetic activity of 4 nM activin A was about 2.5 times higher than that of complete medium (Fig. 1C). A dose-response study revealed that this concentration of...
activin A elicited the maximal effect. As expected, 1 nM FGF-2, an important angiogenetic growth factor, also promoted tube formation of BAEC (Fig. 1A-c). The dose-response study also showed that FGF-2 induced the maximal effect at this concentration. Indeed, activin A was as potent as FGF-2 in inducing tubulogenesis of BAEC. Furthermore, a combination of activin A and FGF-2 dramatically accelerated tube formation of BAEC (Fig. 1A-d). Tubulogenetic activity elicited by the combination of activin A and FGF-2 was statistically significantly higher than that induced by activin A or FGF-2 alone. Thus, the effects of activin A and FGF-2 on tubulogenesis of BAEC were additive. We previously reported that tubulogenesis of BAEC induced by VEGF was totally dependent on the action of activin A produced in these cells [6]. We therefore examined whether or not the effect of FGF-2 was dependent on the action of activin A produced in BAEC. To this end, we examined the effect of follistatin, an antagonist of activin A, on the action of FGF-2. Indeed, follistatin nearly completely inhibited tubulogenetic activity of FGF-2 (Fig. 1B). Consequently, the effect of FGF-2 in BAEC was dependent on the action of endogenous activin A produced in these cells. Collectively, there was a strong interaction between the effects of activin A and FGF-2 in BAEC.

Effect of FGF-2 on the expression of activin A and the type 2 activin receptor in BAEC

We previously showed that VEGF induced the expression of activin A and the activin receptor in BAEC [6], and that activin A in turn augmented the VEGF actions by increasing the expression of the VEGF receptors. These effects were the basis of the modulation of VEGF action by activin A in BAEC [6]. We therefore examined whether FGF-2 induced the production of activin A in BAEC. Measurement of the expression of mRNA for the $\beta_A$ subunit of activin A and the protein expression of activin A revealed that FGF-2 did not affect the production of activin A (data not shown). Instead, FGF-2 increased the expression of the type 2 activin receptor (Fig. 2). The effect of FGF-2 was detected 12 hrs after the addition of FGF-2.

Effect of activin A on the expression of FGF-2 and FGFR1 in BAEC

We then studied the effect of activin A on the expression of FGF-2 and the FGF receptor, FGFR1, in BAEC. As shown in Fig. 3A, quantitative RT-PCR revealed that activin A increased the expression of mRNA for FGF-2. The effect of activin A was detected 1 hr after the addition of activin A, and thus the expression of FGF-2 increased up to 48 hrs. Western blot analysis showed that the intracellular content of FGF-2 was elevated after the treatment of activin A (Fig. 3B). We also measured the concentration of FGF-2 in culture medium by ELISA. The concentration of FGF-2 in the medium treated with activin A for 24 hrs was significantly higher than that treated by FBS alone (Fig. 3C). Activin A also induced the expression of the FGF receptor in BAEC. As shown in Fig. 3D, Western blotting of the FGFR1 revealed that activin A markedly upregulated the expression of FGFR1. The effect of activin A was observed 3 hrs after the addition of activin A and lasted for at least 48 hrs.

These results suggest that activin A facilitates the production of FGF-2 and expression of FGFR1 in BAEC and thereby efficiently promotes the FGF signal for induction of tubulogenesis.

Inhibition of tubulogenesis induced by activin A by overexpression of Smad7

We then addressed the signaling pathways involved in tubulogenesis. We first addressed the involvement of Smads in activin-induced tubulogenesis. To examine whether the promotion of tubulogenesis is Smad-dependent or independent, we transfected BAEC with cDNA encoding Smad7 using adenovirus vector
(AdexSmad7). Since we found that adenovirus at a titer of 10 MOI was efficient in introducing the gene without cytotoxicity [6, 18], we used an adenoviral vector at the titer of 10 MOI in the following studies. When we infected BAEC with AdexSmad7, we were able to detect the expression of Smad7 protein by Western blotting (data not shown).

When cultured in a collagen gel with complete medium, there was no morphological difference between BAEC infected with AdexLacZ and AdexSmad7. In the presence of activin A, BAEC infected with AdexLacZ formed a capillary network, whereas in BAEC infected with AdexSmad7, capillary formation was markedly inhibited (Fig. 4). Quantitative analysis of the tubulogenic activity showed statistically significant reduction of tubulogenic activity in the BAEC infected with AdexSmad7 compared with the BAEC infected with AdexLacZ. Thus, the effect of activin A on tubulogenesis of BAEC is Smad-dependent.

Fig. 3. Effect of activin A on the expression of FGF-2 and FGF receptor
BAEC were incubated for indicated periods in the presence of 4 nM activin A, and the expression of mRNA for FGF-2 was measured by quantitative RT-PCR. Values are the mean of three experiments.
B: Measurement of FGF-2 content in BAEC.
BAEC were incubated for indicated periods with 4 nM activin A, and the FGF-2 content of BAEC was measured by Western blotting. Results are representative of two experiments.
C: Measurement of FGF-2 concentration in medium.
BAEC were incubated for indicated periods with 4 nM activin A, and the FGF-2 concentration of the culture medium was measured by ELISA. Values are the mean ± S.E for 5 experiments.
D: Measurement of the expression of FGFR-1.
BAEC were incubated for the indicated time with 4 nM activin A, and the expression of FGFR-1 was measured by Western blotting.
Suppression of tube formation by p42/p44 MAP kinase inhibitor

We identified that the Smad-pathway is important for the activin A induced tubulogenesis of BAEC. In addition to the Smad-pathway, Smad-independent pathways might be responsible for some of the actions of the TGF-β superfamily ligands. As shown in Fig. 5A, activin A induced phosphorylation of p42/p44 MAP kinase in BAEC. Similarly, activin A induced phosphorylation of p38 MAP kinase (Fig. 5B). To determine the involvement of MAP kinases in tubulogenesis, we incubated BAEC grown in collagen gels with PD98059, a p42/p44 MAP kinase (MEK1) inhibitor, or SB202190, a p38 inhibitor. As observed in Fig. 5C, activin A-induced tubulogenesis was blocked in the presence of PD98059, whereas it was not affected by SB202190. Therefore, activation of p42/p44 MAP kinase might be involved in tubulogenesis of BAEC by activin A.

Discussion

In the present study, we investigated the interaction of activin A and FGF-2 in cultured vascular endothelial cells. Using BAEC as a model system, we determined the tubulogenic activity of these two ligands. In this cell system, FGF-2 induced tube formation as shown in Fig. 1A-c. Activin A also induced tubulogenesis as efficiently as FGF-2. When these ligands were added together, the combination of the two ligands induced further effects. There are multiple steps in the interaction of these two ligands. First, although FGF-2 did not affect the production of activin A in BAEC, FGF-2 increased the expression of the activin receptor. This may have led to upregulation of the activin signal. On the other hand, activin A augmented the production of FGF-2 in BAEC, and secretion of FGF-2 was significantly increased by the activin treatment. In addition, activin A upregulated the expression of the FGF receptor. Collectively, activin A facilitates the FGF signal by increasing both the ligand and its receptor. Hence, there is an autocrine feed-forward loop in BAEC in which FGF-2 and activin A produced in these cells sensitize the tubulogenic action of each other. The significance of this autocrine loop seems to be modest since FGF-2 did not stimulate the production of activin A in BAEC and only increased the receptor expression. Nevertheless, the most impressive finding in this study was that the FGF-2-induced tubulogenesis was nearly completely blocked by an administration of follistatin, an antagonist of activin A. Indeed, this observation suggests that the action of endogenously produced activin A is quite important for the action of FGF-2. The result also suggests that upregulation of the receptor may significantly amplify the activin signal, and that the activin-mediated step is essential for operation of the feed-forward mechanism in BAEC. Presumably, inhibition of the activin action may reduce the autocrine production of FGF-2 and simultaneously the expression of FGF receptor. The autocrine feed-forward loop is thus essential in the FGF-2 action in BAEC.

Previously, we showed that activin A produced in BAEC is critical for VEGF-induced tubulogenesis [6]. Involvement of activin A is slightly different: VEGF increases the production of activin A and simultaneously upregulates the expression of the activin re-
Angiogenesis is an important biological event necessary for organogenesis during development and tissue repair in adults. It is also involved in pathogenesis of many types of diseases [19]. For example, angiogenesis is indispensable for expansion of malignant tumors. Also, angiogenesis plays a major role in the pathophysiology of proliferative retinopathy and rheumatoid arthritis. Consequently, inhibition of angiogenesis by any means is beneficial to treat such disorders. In contrast, promotion of angiogenesis is needed to treat certain ischemic disorders including arterial stenosis and coronary heart diseases. The present results raise the possibility that either activin A or its antagonist follistatin may be useful in promoting or inhibiting angiogenesis in those disorders.

In the present study, we also addressed the intracellular signaling pathway involved in activin-induced tubulogenesis. It is well established that the activin receptors activate the Smad pathway, and the oligomer of Smad2/3 and Smad4 acts as a transcription regulator of various target genes. Smad7, which is also known as an inhibitory Smad, blocks the Smad-mediated transcription. The finding that overexpression of Smad7 in BAEC blocked activin-induced tubulogenesis indicates that the Smad system is also critical in promoting angiogenesis. The results obtained by using PD098005 indicated that the MAP kinase pathway is also important for activin-mediated tubulogenesis. It has been proposed that intracellular signals other than Smad proteins are important for the action of ligands belonging to the TGF-β superfamily [20]. In the case of activin A, transcriptional stimulation of certain target proteins is independent of the Smad pathway [21]. Although the target genes of activin A in BAEC are unknown at present, this is another example of Smad-independent action of activin A.

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References