Inhibition of Vascular Endothelial Cell Growth by Activin-A*

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The demonstration of type 2 activin receptor expression in human umbilical vein endothelial cells prompted an investigation of the effects of the activin/inhibin family of hormones on vascular endothelial cell growth. Recombinant activin-A inhibited [3H]methylthymidine uptake and growth of a panel of endothelial cell types; recombinant inhibin-A was without effect. Affinity cross-linking studies demonstrated the presence of type 1 and type 2 activin receptors on the surface of bovine aortic endothelial cells, while detailed analysis of type 2 activin receptor expression revealed both type 2 and type 2B activin receptor mRNA in all endothelial cell types analyzed. In addition, capillary endothelial cells were found to express activin-ββ subunit mRNA and protein, the levels of which were increased in response to transforming growth factor (TGF)-β. Furthermore, activin-A and TGF-β caused additive inhibition of capillary endothelial cell [3H]methylthymidine uptake. These findings implicate the activins in the regulation of endothelial cell function, and suggest that TGF-β and activin may interact to inhibit capillary endothelial cell growth.

Abnormal endothelial cell proliferation is associated with a number of pathological states, for example, tumor vascularization (Folkman and Shing, 1992). Mechanisms by which endothelial cell growth is controlled are therefore of widespread interest, and although much attention has been paid to the role of endothelial cell mitogens in this respect, it is equally likely that endothelial proliferation could result from a release of inhibitory growth control. Several inhibitors of endothelial cell growth have been identified, including the transforming growth factor-β (TGF-β), interleukin-1, tumor necrosis factor, and platelet factor-4 (Folkman and Shing, 1992; Bicknell and Harris, 1991).

The activins and inhibins were originally characterized as members of the TGF-β superfamily of hormones on vascular endothelial cell growth. Recombinant activin-A inhibited [3H]methylthymidine uptake and growth of a panel of endothelial cell types, as well as inhibition of [3H]methylthymidine uptake of a panel of endothelial cell types analyzed. In addition, capillary endothelial cells were found to express activin-ββ subunit mRNA and protein, the levels of which were increased in response to transforming growth factor (TGF)-β. Furthermore, activin-A and TGF-β caused additive inhibition of capillary endothelial cell [3H]methylthymidine uptake. These findings implicate the activins in the regulation of endothelial cell function, and suggest that TGF-β and activin may interact to inhibit capillary endothelial cell growth.

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The abbreviations used are: TGF-β, transforming growth factor-β; BAEC, bovine adrenal capillary endothelial cells; CPAE, calf pulmonary artery endothelial cells; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; FGF, basic fibroblast growth factor; FCS, fetal calf serum; ACTR, activin receptor.

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High affinity cell surface receptors for activin have been identified on a number of gonadal and non-gonadal cell types (Campen and Vale, 1988; Sugino et al., 1988; Hino et al., 1989; Kondo et al., 1989; Centrella et al., 1991; Shao et al., 1992). By analogy to the TGF-β receptor system, these can be classified as type 1 and type 2 receptors (Roberts and Sporn, 1990; Massague, 1990). Two subtypes of the type 2 activin receptor have recently been characterized by cDNA cloning and shown to define a novel class of transmembrane receptor proteins, the putative receptor serine-threonine kinases (Mathews and Vale, 1991; Attisano et al., 1992; Kondo et al., 1991; Mathews et al., 1992; Legerski et al., 1992). A type 2 TGF-β receptor has also recently been characterized by cDNA cloning, and, as predicted by cross-linking studies, shown to belong to the same receptor family as the type 2 activin receptors (Lir et al., 1992). Thus, it is possible that all members of the TGF-β superfamily employ receptors of this class to elicit cellular responses (Massague, 1992).

In addition to regulation of reproductive function, the activins/inhibins regulate many other cellular processes. Activin-A is identical to erythrocyte differentiation factor, a potent stimulator of red blood cell formation (Eto et al., 1987; Yu et al., 1987), influences cells of the central nervous system (Sawchenko et al., 1988; Schubert et al., 1990), induces mesoderm and axial structures in early vertebrate development (Smith et al., 1990; van der Eijnden-Van Raaij et al., 1990; Mitrani et al., 1990; Thomsen et al., 1990; Hemmati-Brivanlou and Melton, 1992) and cooperates with bone morphogenetic proteins to promote bone formation (Ogawa et al., 1992). We report here a study of the role of activin-A in regulation of endothelial cell growth, prompted by the finding that human umbilical vein endothelial cells express the type 2 activin receptor, ACTR2.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human activin-A and inhibin-A were a gift of Genentech Inc., San Francisco, CA. All radiolabels were from Amersham Corp. TGF-β1 was from British Biotechnology Ltd., Oxford, UK. T3 RNA polymerase was from Bohringer Mannheim, Mannheim, Germany. T3 RNA polymerase from Life Technologies, Inc. Disuccinimidyl suberate was from Pierce Chemical Co. Anti-activin-β subunit monoclonal antibody (Groome and Lawrence, 1991) was the gift of Professor Nigel Groome, Oxford-Brookes University, Oxford, UK. All tissue culture media were from ICRF Central Services.

Cell Isolation and Culture—Human umbilical vein endothelial cells were isolated from fresh umbilical cords by dissociation with a 20% solution of "Dispase" (Collaborative Research Ltd.) in phosphate-buffered saline and used between passage 2 and 4. Bovine adrenal capillary endothelial cells were isolated and characterized as described.
elsewhere (Fawcett et al., 1991; McCarthy and Bicknell, 1992). HUVECs and BACE were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, heparin (50 μg/ml), and basic FGF (1 ng/ml). Bovine aortic endothelial cells were isolated from fresh bovine aorta by collagenase digestion followed by fluorescence-activated cell sorting of the resultant cell population using different antibodies (Hennessy et al.) as an endothelial marker (Voyta et al., 1984). Calf pulmonary artery endothelial cells were from the American Type Culture Collection, Bethesda, MD (ATCC CCL 205). BAEC and CPAE were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. All endothelial cells were cultured in Petri dishes coated with a solution of 0.1% gelatin in phosphate-buffered saline.

**Human ACTR2 Cloning**—Human ACTR2 was amplified from both HUVEC and human placenta cDNA libraries using the following oligonucleotide primers: forward primer, 5'-ACCCAGGAACTGGACTTCACTCT-3', representing nucleotides 12-33 and 1710-1691, respectively, of the murine type 2 activin receptor sequence (Mathews and Vale, 1991). The polymerase chain reaction was for 40 cycles at an annealing temperature of 40°C. The 1.7-kb product was ligated into pBluescript, and sequenced by the double-stranded dyeoxynucleotide chain termination protocol.

**51Methylthymidine Uptake Assays and Growth Curves**—For thymidine uptake studies, endothelial cells were plated in 96-well gelatin coated plates at 1000 cells/well in 10% FCS. Four hours later, cells were treated as indicated in either 10% FCS (BACE, HUVEC, CPAE) or 0.5% FCS for 24 h, cells were washed with 1 mM disuccinimidyl suberimidate for 3 h, trypsinized, and harvested using an automated 96-well plate harvester (Pharmacia-Wallac). Counts were determined using a flat-bed 96 plate counter. For growth curves, cells were plated in gelatin coated 6-well plates at 11,000 cells/well (HUVEC), 12,000 cells/well (BACE, CPAE), or 29,000 cells/well (BAEC) in 10% FCS (CPAE), 10% FCS + 1 ng/ml basic FGF (BACE), 10% FCS + 1 ng/ml basic FGF (HUVEC), or 0.5% FCS (BAEC) and treated as indicated for 6 days. Fresh medium containing the appropriate factor was applied on days 2 and 4. Cell numbers were determined on days 0, 3, and 6 using a Coulter counter.

**Activin Receptor Expression in Endothelial Cells**—The nature of the receptor system mediating growth inhibitory actions of activin-A on vascular endothelial cells was subsequently investigated. Affinity cross-linking of 125I-activin-A to BAEcs using the bifunctional cross-linking reagent, disuccinimidyl suberate, revealed the presence of three activin binding proteins of 160, 80, and 65 kDa, binding to each of which was fully competed by unlabeled activin-A (Fig. 3a). Competition was complete in the presence of 25 ng/ml (0.9 nM) unlabeled activin-A, in close agreement with the affinity of the cross-linked activin-receptor complexes for activin-A demonstrated in Fig. 3a. Consistent with inhibition of endothelial cell DNA synthesis, activin-A caused growth inhibition of HUVEC, BACE, CPAE, and BAEC (Fig. 2). Activin-A did not affect cell attachment in any experiment. As with [3H]methylthymidine uptake, inhibin-A did not antagonize inhibition of endothelial cell growth by activin-A (data not shown).

**Activin Receptor Expression in Endothelial Cells**—The nature of the receptor system mediating growth inhibitory actions of activin-A on vascular endothelial cells was subsequently investigated. Affinity cross-linking of 125I-activin-A to BAEcs using the bifunctional cross-linking reagent, disuccinimidyl suberate, revealed the presence of three activin binding proteins of 160, 80, and 65 kDa, binding to each of which was fully competed by unlabeled activin-A (Fig. 3a). Competition was complete in the presence of 25 ng/ml (0.9 nM) unlabeled ligand. Subtraction of the molecular mass of the cross-linked activin-βA subunit yields receptor molecular masses of approximately 50, 65, and 145 kDa. By analogy to the TGF-β receptor system, the 45- and 60-kDa species represent types 1 and 2 activin receptors, respectively (Massague, 1992). The identity of the 145-kDa component is unclear.

**RESULTS**

**Effect of Activin-A on Endothelial Cell Growth**—The finding that prompted this series of experiments was the cloning of a full-length cDNA encoding a type 2 activin receptor (Mathews and Vale, 1991) from a human umbilical vein endothelial cell (HUVEC) cDNA library by polymerase chain reaction amplification (data not shown). Sequence analysis of the HUVEC activin receptor revealed complete identity with human ACTR2 cDNAs isolated from testis (Donaldson et al., 1992; Matzuk and Bradley, 1992). Since activin-A exerts diverse effects on cellular growth and differentiation, ranging from induction of mesoderm during embryogenesis to stimulation of erythroid development, the effects of activin-A/inhibin-A on endothelial cell growth were subsequently investigated.

Recombinant human activin-A caused dose-dependent inhibition of [3H]methylthymidine incorporation into DNA in four endothelial cell types, namely BACE, CPAE, BAEC, and HUVEC (Fig. 1). The dose dependence of the inhibition for each cell type was similar, with half-maximal inhibition observed between 1 and 10 ng/ml (36 and 360 pM, respectively). Recombinant human inhibin-A had no effect on endothelial cell [3H]methylthymidine incorporation, neither did inhibin-A antagonize the growth inhibitory effects of activin-A (data not shown). To confirm that inhibition of [3H]methylthymidine uptake reflected growth inhibition, the effect of activin-A on endothelial cell growth was assessed over a 6-day period. Cells were treated every 2 days with activin-A at the indicated concentration. Consistent with inhibition of endothelial cell DNA synthesis, activin-A caused growth inhibition of HUVEC, BACE, CPAE, and BAEC (Fig. 2). Activin-A did not affect cell attachment in any experiment. As with [3H]methylthymidine uptake, inhibin-A did not antagonize inhibition of endothelial cell growth by activin-A (data not shown).
Fig. 1. Inhibition of endothelial cell DNA synthesis by activin-A. 
[3H]Methylthymidine incorporation into DNA in endothelial cells treated
with the indicated concentrations of human recombinant activin-A for 24 h. a, HUVEC; b, BACE; c, CPAE; d, BAEC. 
The y axis scales are adjusted to account for the different basal levels of thymidine uptake in the different cell lines (mean ± S.E., n = 12).

Fig. 2. Inhibition of endothelial cell growth by activin-A. Growth of endothelial cells over a 6-day period after plating of identical numbers of cells in six-well plates. Filled bars, no addition; hatched bars, + human recombinant activin-A (10 ng/ml, HUVEC, BACE, and CPAE, 50 ng/ml, BAEC) a, HUVEC; b, BACE; c, BAEC; d, CPAE. Statistical significance was determined using the paired t-test.

Actinin-βA Expression in Capillary Endothelial Cells—Immunohistochemical analysis of actinin-βA subunit expression in a panel of human tissues revealed granular cytoplasmic staining of endothelium in vessels of many organs, suggesting that in addition to responding to activin-A, vascular endothelial cells also express this ligand. Expression of actinin-βA mRNA in capillary endothelial cells (BACE) was therefore investigated. As shown in Fig. 5, actinin-βA mRNA was expressed at a low level in BACE cells treated with control

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FIG. 3. Binding of $^{125}$I-activin-A to bovine aortic endothelial cells. a, affinity cross-linking of $^{125}$I-activin-A to BAEC. Confluent cultures of BAEC were exposed to $^{125}$I-activin-A (2 ng/ml) in the presence of the indicated concentration of unlabelled activin-A, and bound ligand subsequently chemically cross-linked using disuccinimidyl suberate. The positions of type 1 and type 2 activin receptors are indicated. The lower band represents cross-linked (β₁)₂ dimer, b, ligand specificity of BAEC activin receptors. Competition of $^{125}$I-activin-A (2 ng/ml) binding to BAEC by the indicated concentrations of activin-A (circles), inhibin-A (squares), and TGF-β (triangles). Specific binding was 60% of total binding.

FIG. 4. Type 2 activin receptor expression in vascular endothelial cells. RNase protection analysis of activin receptor subtype mRNA expression in endothelial cells. a, ACTR2; b, ACTR2B. Lane 1, BAEC; lane 2, CPAE; lane 3, BACE; lane 4, HUVEC; lane 5, human placenta control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) represents a loading control. These data are representative of three similar experiments. The weak signals from HUVEC result from the use of bovine-specific probes in the experiments shown. In experiments using human-specific probes, strong signals for both ACTR2 and ACTR2B were obtained from HUVEC RNA (data not shown).

FIG. 5. ACTR2 and ACTR2B were obtained from HUVEC RNA (data not shown). FIG. 6. Activin-βₐ subunit mRNA expression in capillary endothelial cells. RNase protection analysis of activin-βₐ mRNA in BACE cells treated for 24 h in Dulbecco’s modified Eagle’s medium + 10% FCS with the following additions: lane 1, no addition; lane 2, basic FGF (10 ng/ml); lane 3, TGF-β1 (10 ng/ml); lane 4, TGF-β2 (10 ng/ml); lane 5, interleukin-1α (10 ng/ml); lane 6, TNF-α (10 ng/ml); lane 7, human placenta control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) represents a loading control. These data are representative of three similar experiments.

FIG. 7. Activin-βₐ protein release from capillary endothelial cells. Western analysis of activin-βₐ subunit protein in BACE cell conditioned medium. BACE cells were treated with either control medium (lane 1) or medium containing TGF-β1 (10 ng/ml; lane 2) for 72 h. Conditioned medium was collected and analyzed as described under “Experimental Procedures.” Lane 3 contained 50 ng of recombinant human activin-βₐ for comparison. Similar results were obtained in three separate experiments.

medium (lane 1). Cells, treated with medium containing the endothelial cell mitogen basic FGF, lacked detectable activin-βₐ mRNA (lane 2). Treatment of BACE cells with either TGF-β1 (lane 3) or TGF-β2 (lane 4) caused a substantial increase in activin-βₐ mRNA levels, however. Treatment with two other endothelial cell growth inhibitors, TNF-α (lane 5) and IL-1α (lane 6), had no effect on βₐ mRNA levels. Thus, capillary endothelial cells express activin-βₐ subunit, expression of which is stimulated specifically by TGF-β. In similar experiments, inhibin-α subunit mRNA was not detectable in BACE cells, either by RNase protection or polymerase chain reaction of reverse-transcribed mRNA (data not shown).

Expression of activin-βₐ protein by BACE cells was investigated. Confluent cultures were treated either with control medium or with TGF-β1 (10 ng/ml) for 72 h, following which conditioned medium was collected and analyzed for activin-βₐ subunit protein by Western blotting. As shown in Fig. 6, untreated BACE cells secreted mature 14-kDa activin-βₐ subunit at a low level (lane 1). TGF-β1-treated cells displayed an approximately 2-fold increase in secreted activin-βₐ subunit (lane 2), confirming that TGF-β regulated expression of activin-βₐ mRNA results in increased secretion of mature activin-βₐ protein from BACE cells.

Effect of Activin-A and TGF-β1 on Capillary Endothelial
Cell DNA Synthesis—Taken together with the lack of detectable inhibin-α mRNA expression in BACE cells, the ability of TGF-β to stimulate activin-βA expression pointed to TGF-β as a stimulator of activin release from capillary endothelial cells. The effect of simultaneous addition of activin-A and TGF-β1 on BACE cells [3H]methylthymidine uptake was therefore investigated to determine whether these two related molecules can interact to regulate capillary endothelial cell growth (Fig. 7). TGF-β1 caused dose-dependent inhibition of BACE cell [3H]methylthymidine uptake, as previously described (McCarthy and Bicknell, 1992). At each concentration of TGF-β1, activin-A at both 5 and 50 ng/ml caused increased inhibition of BACE cell DNA synthesis compared to TGF-β1 alone. These data suggest that TGF-β-stimulated release of activin-A may augment the growth inhibitory response to TGF-β.

DISCUSSION

The TGF-β superfamily is comprised of several homologous growth regulatory molecules that exert diverse effects on cellular growth and differentiation, many of which are shared by more than one family member. Identification by cDNA cloning of type 2 receptors for activin and TGF-β as transmembrane serine-threonine kinases has shed light for the first time on signaling mechanisms employed by these pleiotropic molecules (Massague, 1992) and permitted analysis of their expression in diverse cell types. The identification of type 2 activin receptor expression in HUVECs suggested for the first time that the activin/inhibin family of peptide hormones may regulate endothelial cell function. We have therefore investigated the relevance of the activins/inhibins to endothelial cell biology using a representative panel of endothelial cell types.

Although first isolated and characterized in the context of paracrine and endocrine actions on cells of the reproductive system (De Jong, 1988; Vale et al., 1990), the activins and inhibins are now known to be expressed in a variety of non-gonadal tissues (Meunier et al., 1988), and are becoming increasingly recognized as regulators of diverse cellular processes. In common with TGF-β, activin-A can either stimulate or inhibit DNA synthesis depending upon the cell type (Kojima and Ogata, 1989; Hedger et al., 1989; Gonzalez-Manchon and Vale, 1989; Centrella et al., 1991; Shao et al., 1992). Investigation of the effect of activin-A on endothelial cell [3H]methylthymidine incorporation revealed potent inhibition of DNA synthesis in four endothelial cell types. These findings were extended to endothelial cell growth, which in each cell type was decreased by activin-A. Thus, in common with the TGF-βs, activin-A is an inhibitor of vascular endothelial cell growth. Inhibin-A had no effect on endothelial cell growth, nor did it antagonize the inhibitory effect of activin-A.

Cross-linking of 125I-activin-A revealed three activin-binding proteins on the surface of BACEs, the sizes of which were similar to those observed on other activin-responsive cell lines (Hino et al., 1989; Centrella et al., 1991; Shao et al., 1992). By analogy to the TGF-β receptor system, two of these receptors were interpreted as type 1 (50 kDa), and type 2 (65 kDa) components (Roberts and Sporn, 1990; Massague, 1990, 1992; Wrana et al., 1992). The identity of the 145-kDa receptor component remains to be established, although, again by analogy to the TGF-β receptor system, this may represent a type 3 activin receptor (Lopez-Casillas et al., 1991; Wang et al., 1991). Specific binding of 125I-activin-A to BACEs was competed fully by activin-A, partially competed by inhibin-A, and unaffected by TGF-β1. In experiments to address whether inhibin-A could antagonize the action of activin-A on endothelial cells, as observed in other cell types (De Jong et al., 1988; Vale et al., 1990), a 2-fold excess of inhibin-A over activin-A had no effect on activin-A-mediated growth inhibition of CPAE, BACE or HUVEC. However, since competitive activin-A binding required a 50-fold excess of inhibin-A (Fig. 3b), it remains possible that higher inhibin concentrations may be effective.

Although a low level of 125I-activin-A binding to CPAE could also be detected, no binding could be obtained to either BACE or HUVEC. Thus, as with the TGF-βs, not all activin-responsive cell types readily display ligand binding (Wrana et al., 1992). However, expression of both ACTR2 (Mathews and Vale, 1991) and ACTR2B (Attia et al., 1992) mRNAs was subsequently demonstrated in all four endothelial cell types used in this study, consistent with responsiveness of each cell type to activin-A, and with type 2 activin receptors being essential signal generating components of the activin receptor system (Massague, 1992; Wrana et al., 1992).

Endothelial cells are known to synthesize a number of polypeptide growth factors to which they respond, for example basic FGF (Schweiger et al., 1987) and TGF-β1 (Antonelli-Oridge et al., 1989; Sato and Rifkin, 1989; McCarthy and Becknell, 1992). Investigation of expression of the activins/inhibins in capillary endothelial cells (BACE) revealed low level expression of activin-βA mRNA in the absence of added factors. Moreover, activin-βA mRNA expression in BACE cells was stimulated specifically in response to TGF-β1 or TGF-β2, and suppressed in the presence of PGF. Activin-βA mRNA expression is known to be regulated by various stimuli in other cell types (Takahashi et al., 1990, 1992; Yamashita et al., 1992). Mechanisms responsible for the regulation of activin-βA mRNA expression described here or elsewhere remain to be established.

Analysis of activin-βA protein expression in BACE cells confirmed that TGF-β1 stimulates activin-βA protein secretion into BACE conditioned medium. Thus, both mRNA and protein data indicate that TGF-β stimulates activin-βA expression in BACE cells. Since expression of inhibin-α subunit mRNA was not detected in BACE cells, it is assumed that this reflects TGF-β stimulated release of activin. In addition to stimulation of activin release, TGF-β1 also stimulates its own expression in capillary endothelial cells (McCarthy and Bicknell, 1993). Interestingly, simultaneous addition of TGF-β and activin-A to BACE cells caused additive

8. S. McCarthy, unpublished results.
inhibition of DNA synthesis, suggesting that TGF-β may promote its own inhibitory effects by induction of activin-A in addition to TGF-β. Further work is required to formally prove whether activin-A mediates any component of TGF-β growth inhibition, and to determine whether this mechanism operates in other cell types. It will also be interesting to determine whether capillary endothelial cells express other TGF-β superfamily members, and if so, whether these are similarly regulated by TGF-β. Recent work has established that BACE cells express mRNAs encoding the bone morphogenetic proteins BMP-2 and BMP-4. Future studies will be aimed at understanding how these and other factors interact to regulate vascular endothelial cell growth.

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