Communication

Endoglin Is a Component of the Transforming Growth Factor-β Receptor System in Human Endothelial Cells*

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Endoglin, a dimeric membrane glycoprotein expressed at high levels on human vascular endothelial cells, shares regions of sequence identity with betaglycan, a major binding protein for transforming growth factor-β (TGF-β) that co-exists with TGF-β receptors I and II in a variety of cell lines but is low or absent in endothelial cells. We have examined whether endoglin also binds TGF-β and demonstrate here that the major TGF-β1-binding protein co-existing with TGF-β receptors I and II on human umbilical vein endothelial cells is endoglin, as determined by specific immunoprecipitation of endoglin affinity-labeled with 125I-TGF-β. Furthermore, endoglin ectopically expressed in COS cells binds TGF-β1. Competition affinity-labeling experiments showed that endoglin binds TGF-β1 (Kp ~ 50 pm) and TGF-β3 with high affinity but fails to bind TGF-β2. This difference in affinity of endoglin for the TGF-β isoforms is in contrast to betaglycan which recognizes all three isoforms. TGF-β however is binding with high affinity to only a small fraction of the available endoglin molecules, suggesting that some rate-limiting event is required to sustain TGF-β binding to endoglin.

Endoglin is a homodimeric membrane glycoprotein composed of disulfide-linked subunits of 95 kDa (1, 2). It is expressed on human pre-erythroblasts, macrophages, leukemic cells of the lymphoid and myeloid lineages, and at higher levels on syncytiotrophoblasts of term placenta and vascular endothelial cells (1, 3–5). A relationship between human endoglin and TGF-β3 receptor system was discovered with the molecular cloning of the rat TGF-β-binding proteoglycan, betaglycan (also known as the type III TGF-β receptor), which revealed that the transmembrane domain and the relatively short (43 amino acids) cytoplasmic tail of this protein (6, 7) were remarkably similar (71% amino acid sequence similarity with 63% identity) to the corresponding regions in endoglin (1) (Fig. 1). The extracellular domains of these two proteins show limited amino acid sequence homology (1, 6–8), and although endoglin contains O-linked oligosaccharides, it is not a proteoglycan (9). Endoglin contains an RGD sequence and so is potentially involved in RGD-mediated cellular adhesion (5), whereas betaglycan does not contain this sequence.

In addition to betaglycan, the TGF-β receptor system (10) in most mesenchymal and epithelial cells consists of the type I receptor, a 53-kDa glycoprotein whose structure has not been determined yet (10), and the type II receptor (11), which belongs to the protein serine/threonine kinase receptor family (10–14). Additional cell surface TGF-β-binding proteins, some of which have a more restricted distribution, have also been described (15–17). In particular, endothelial cells which express the TGF-β receptors I and II but have little or no betaglycan (17–20) have been shown to express a disulfide-linked protein dimer of 95-kDa subunits, which binds TGF-β1 but not TGF-β2 (17). The size and restricted distribution of this TGF-β-binding protein is remarkably similar to that of endoglin. These observations in conjunction with the struct-

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††† The abbreviations used are: TGF-β, transforming growth factor-β; DTT, dithiothreitol; HUVEC, human umbilical cord endothelial cells(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

FIG. 1. Domain structures of betaglycan and endoglin. Schematic representation highlighting regions of similarity between the linear sequences of betaglycan, an 853-amino acid transmembrane proteoglycan (6, 7), and endoglin, a disulfide-linked transmembrane protein composed of subunits of 633 amino acids (1). The transmembrane and short cytoplasmic regions (dark shaded box) of endoglin have a high level of sequence similarity to the corresponding regions of betaglycan. Two regions of weaker similarity are detected in the ectodomains of these proteins (light shaded boxes). Numbers represent the percent amino acid sequence similarity between the indicated domains of betaglycan and endoglin. Closed ovals represent position of cysteine residues. Two putative sites for glycosaminoglycan chain attachment in betaglycan (6) are indicated.
tural relationship between endoglin and betaglycan prompted an examination of whether endoglin binds TGF-β. We have used monoclonal antibodies specific to human endoglin and an endoglin expression vector to demonstrate that endoglin is a major TGF-β-binding protein in human vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Human umbilical vein endothelial cells (HUVEC, CRL 1730, ATCC) were maintained in α-minimal essential media supplemented according to supplier's instructions or prepared from umbilical veins as previously described (2). Similar results were obtained using cells from either source. COS-M6 cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum, were transfected with a cDNA encoding full-length endoglin (1) ligated into the EcoRI site of the mammalian expression vector pCEXV (21), or with a control vector without cDNA insert (pCMV5; Ref. 6) by the DEAE-dextran-chloroquine procedure (22). 24 h post-transfection, cells were trypsinized and reseeded into multicenter dishes and allowed to grow an additional 48 h before being affinity-labeled with [125I]-TGF-β1 as described below.

Receptor Affinity Labeling and Immunoprecipitations—TGF-β1 and TGF-β2 were purchased from R & D Systems (Minneapolis, MN), and TGF-β3 was obtained from Oncogene Science. 125I-TGF-β1 used in these studies was prepared by the chloramine-T method as previously described (23) or purchased from Amersham Corp.; both preparations gave identical results. The conditions for affinity labeling cell monolayers with 125I-TGF-β1 and disuccinimidyl carbonate (Pierce Chemical Co.) have been described previously (24). The concentrations of 125I-TGF-β1 and competiting unlabeled ligands used for each experiment are indicated in the figure legends. Triton X-100 extracts of the affinity-labeled cells were either analyzed directly on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) or first incubated with monoclonal antibody (mAb) 44G4 directed against human endoglin (25) or with control antibody (see below). For immunoprecipitations, detergent extracts were diluted with an equal volume of phosphate-buffered saline containing 1% Triton X-100 and precleared by incubation for 20 min at 4 °C with protein G-Sepharose (Pharmacia LKB Biotechnology Inc.) prior to overnight incubation at 4 °C with mAb 44G4. Immunocomplexes were collected by incubation with protein G-Sepharose for 1 h at 4 °C. For some experiments, mAb 44G4 was used coupled to Sepharose (25). The immunoprecipitate was washed three times (saline with 1% Triton X-100) and then resolved by SDS-PAGE in the presence or absence of dithiothreitol (DTT) and visualized by autoradiography. Irrelevant mAb (44D7) (25) used in control experiments to monitor specificity of the immunoprecipitations did not immunoprecipitate any affinity-labeled bands (data not shown).

RESULTS

Analysis of the affinity-labeled profile of HUVEC revealed that like vascular endothelial cells from other sources, these cells have little or no betaglycan, which characteristically migrates as a diffuse band between 200 and 400 kDa on reducing SDS-PAGE (Fig. 2A). Instead, HUVEC expressed a disulfide-linked cell surface protein that, together with TGF-β receptors I and II, was affinity-labeled by cross-linking with 125I-TGF-β1. Receptors I and II were detected in HUVEC as labeled complexes of approximately 65 and 100 kDa, which is similar to the size of these labeled receptors reported for other human cell lines (26). The disulfide-linked TGF-β1-binding protein migrated as a labeled complex at 180 kDa and above on nonreducing SDS-PAGE gels while on reducing gels it migrated as a labeled 110-kDa species that overlapped the labeled type II receptor band (Fig. 2A).

Resolution of these disulfide-linked TGF-β1-binding proteins on two-dimensional gels (Fig. 2B) confirmed that the disulfide-linked complexes (probably dimers and higher order oligomers) contained subunits of approximately 95 kDa (value estimated by subtracting the cross-linked TGF-β1 monomer mass, 12.5 kDa from the reduced 110-kDa affinity-labeled complex). Together with the type II receptor, the disulfide-linked TGF-β1-binding proteins are the major affinity-labeled species expressed by HUVEC.

Given: 1) the similarity in subunit composition between this TGF-β-binding protein and endoglin, 2) the presence of endoglin on endothelial cells, and 3) the structural relationship between endoglin and betaglycan, we sought to determine whether this disulfide-linked TGF-β-binding protein on endothelial cells is indeed endoglin. To this end, affinity-labeled HUVEC extracts were immunoprecipitated with monoclonal antibody 44G4, which is specific for human endoglin (1, 2, 25). Electrophoretic analysis of these immunoprecipitates revealed a labeled protein complex whose subunit structure was similar to that of endoglin (Fig. 3A). Thus under reducing conditions, a major affinity-labeled band of approximately 110 kDa was seen which migrated as complexes of 180 kDa and greater than 200 kDa when analyzed under nonreducing conditions. The higher order oligomers might contain multiple endoglin molecules cross-linked by TGF-β1, itself a disulfide-linked dimer (27). Repeated immunoprecipitation with 44G4-IgG-Sepharose completely depleted these labeled species from cell extracts (Fig. 3B). No affinity-labeled bands were immunoprecipitated from three other human cell lines (A549, Hep G2, MCF-7), which lack endoglin and were used as negative-controls for these experiments (data not shown).

The identity of this dimeric TGF-β-binding protein of HUVEC with endoglin was confirmed by ectopically expressing the endoglin cDNA in COS monkey kidney cells. After affinity-labeling with 125I-TGF-β1, a labeled species with the characteristics of endoglin could be specifically precipitated by mAb 44G4 only from detergent extracts of endoglin-transfectants (Fig. 4). Differences in glycosylation likely account for the smaller size of endoglin expressed in COS cells relative to endogenous endoglin of HUVEC.

The TGF-β isoform binding specificity of endoglin was assessed by testing the ability of TGF-β1, -β2, and -β3 to

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were analyzed under reducing conditions on protein G-Sepharose. After washes, equal aliquots of the samples were incubated with mAb 44G4 and immune complexes were collected by SDS-PAGE (5-8% polyacrylamide gradient gels) as described in Fig. 2. HUVECs were affinity-labeled with 100 pM ^251-TGF-β1 complexes. HUVECs were affinity-labeled with 100 pM ^251-TGF-β1 and the detergent extracts of affinity-labeled cells were incubated with mAb 44G4 and immune complexes were collected by SDS-PAGE (5-8% polyacrylamide gradient gels). The migration positions of TGF-β receptor endoglin monomer, dimer, and oligomer are indicated. Thus, endoglin is identified here as a major TGF-β binding molecule.

**DISCUSSION**

The present studies demonstrate that one function of endoglin is to bind TGF-β in an isoform-restricted manner. Thus, endoglin is identified here as a major TGF-β binding glycoprotein in human umbilical vein endothelial cells. The disulfide-linked dimeric TGF-β-binding protein previously identified on fetal bovine heart endothelial cells (17) is likely to be bovine endoglin, although this point could not be proven in the present studies because of the lack of appropriate antibodies. Given the presence of endoglin in human pre-erythroblasts, macrophages, lymphoid, and myeloid leukemic cells, and placental syncytiotrophoblasts, it will be of interest to determine whether various previously described TGF-β-binding proteins of size and TGF-β binding properties similar to those of endoglin (28, 29) might indeed correspond to this molecule.

It is important to note that the molecular mass and electrophoretic migration of the reduced endoglin monomer and the human TGF-β receptor II, as well as their ability to bind TGF-β1 and β3 better than β2, are similar. However, the primary structure and presumed functional role of endoglin and the TGF-β receptor II are very different. Therefore, conventional receptor affinity-labeling procedures alone are insufficient to distinguish between endoglin and the TGF-β receptor II in studies of the expression, function, and regulation of these two molecules. Additional reagents, such as antibodies to TGF-β receptor II, will be essential for such studies.

The results also indicate that only a portion of the endoglin
molecules expressed in HUVEC bind TGF-\(\beta\) with high affinity. The rate-limiting event required to sustain high affinity TGF-\(\beta\) binding to endoglin is unknown at present. It is possible that a post-translational modification of endoglin, a conformational change or an interaction with another cellular component may be required to generate the high affinity TGF-\(\beta\) binding site in endoglin. The nature of this event is currently under investigation.

The specific role of endoglin as a mediator of cell interaction with TGF-\(\beta\) remains to be determined. The homology between the transmembrane and cytoplasmic portions of endoglin and betaglycan suggests that they might associate through these regions with similar molecules in order to fulfill their function(s). As no signal transducing structure is discernible in the cytoplasmic domain of either endoglin or betaglycan, for these proteins to be involved in signaling, they must interact with specific signaling components. In the TGF-\(\beta\) system, the evidence suggests that TGF-\(\beta\) signaling is primarily mediated by TGF-\(\beta\) receptors I and II (10), and as previously proposed for betaglycan (6, 7), endoglin may be involved in presenting TGF-\(\beta\) to these signaling receptors or participate as an accessory molecule in the TGF-\(\beta\) receptor signaling complexes. We note that the TGF-\(\beta\) isoform binding specificity of endoglin is remarkably similar to the profile of TGF-\(\beta\) responsiveness in endothelial cells; TGF-\(\beta 1\) and \(\beta 3\) are strong inhibitors of proliferation of endothelial cells from various sources (23, 30, 31), whereas TGF-\(\beta 2\) is not (20, 23, 31). Endoglin, and its relative betaglycan, might act as modulators of TGF-\(\beta\) interaction with the signaling receptors, I and/or II, thus affecting the ability of cells to respond to the TGF-\(\beta\) isoforms.

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