Endoglin Modulates Cellular Responses to TGF-β1

Pedro Lastres,* Ainhoa Letamendía,* Hongwei Zhang,‡ Carlos Rius,* Nuria Almendro,* Ulla Raab,* Luis A. López,§ Carmen Langa,* Angels Fabra,§ Michelle Letarte,‡ and Carmelo Bernabéu*

*Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), 28006 Madrid, Spain; ‡Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto, Canada M5G 1X8; and §Cancer Research Institute, Hospital Duran i Reynals, Barcelona 08907, Spain

Abstract. Endoglin is a homodimeric membrane glycoprotein which can bind the β1 and β3 isoforms of transforming growth factor-β (TGF-β). We reported previously that endoglin is upregulated during monocyte differentiation. We have now observed that TGF-β itself can stimulate the expression of endoglin in cultured human monocytes and in the U-937 monocytic line. To study the functional role of endoglin, stable transfectants of U-937 cells were generated which overexpress L- or S- endoglin isoforms, differing in their cytoplasmic domain. Inhibition of cellular proliferation and downregulation of c-*myc* mRNA which are normally induced by TGF-β1 in U-937 cells were totally abrogated in L-endoglin transfectants and much reduced in the S-endoglin transfectants. Inhibition of proliferation by TGF-β2 was not altered in the transfectants, in agreement with the isoform specificity of endoglin. Additional responses of U-937 cells to TGF-β1, including stimulation of fibronectin synthesis, cellular adhesion, platelet/endothelial cell adhesion molecule 1 (PECAM-1) phosphorylation, and homotypic aggregation were also inhibited in the endoglin transfectants. However, modulation of integrin and PECAM-1 levels and stimulation of mRNA levels for TGF-β1 and its receptors R-I, R-II, and betaglycan occurred normally in the endoglin transfectants. No changes in total ligand binding were observed in L-endoglin transfectants relative to mock, while a 1.5-fold increase was seen in S-endoglin transfectants. The degradation rate of the ligand was the same in all transfectants. Elucidating the mechanism by which endoglin modulates several cellular responses to TGF-β1 without interfering with ligand binding or degradation should increase our understanding of the complex pathways which mediate the effects of this factor.

TRANSFORMING growth factor-β (TGF-β) is a member of a large family of proteins that has many biological effects including regulation of cellular proliferation, differentiation and migration, extracellular matrix formation, and modulation of the immune response (Massagué et al., 1994; Roberts and Sporn, 1993; Kingsley, 1994). In humans, three isoforms have been identified, namely TGFβ1, TGFβ2, and TGFβ3. TGFβs exert their function through binding to specific receptors, including receptors type I (R-I), type II (R-II), betaglycan, and endoglin (Kingsley, 1994; Attisano et al., 1994; Miyazono et al., 1994; Yingling et al., 1995; López-Casillas et al., 1991; Cheifetz et al., 1992). Among these, the serine-threonine kinase receptor types I and II are necessary for all tested biological responses to TGF-β (Attisano et al., 1994; Miyazono et al., 1994; Yingling et al., 1995; Franzen et al., 1993; Bassing et al., 1994; Wrana et al., 1994; Laiho et al., 1991; Wrana et al., 1992; Cárcamo et al., 1994; Koenig et al., 1994). Although the molecular mechanism of receptor activation is rather complex, recent data by Wrana et al. (1994) have provided some hints about the receptor I/receptor II interplay. Thus, TGF-β binding to the constitutively phosphorylated R-II is followed by recruitment of R-I into the complex, phosphorylation of R-I and propagation of the signal to downstream substrates (for a recent review see Yingling et al., 1995).

Betaglycan forms heteromeric complexes with the signaling receptors type I and II and may function as a regulator of TGF-β access to the signaling heteromeric kinase receptor complex formed by TGF-β receptor components I and II (López-Casillas et al., 1993; Moustakas et al., 1993). It has been demonstrated that betaglycan increases the TGF-β binding to the receptor II, which then recruits type I into the complex (López-Casillas et al., 1993). Little is known about the possible role of endoglin in the TGF-β signaling pathways, although the heteromeric association between endoglin and the signaling receptors has been suggested by coimmunoprecipitation experiments (Yamashita et al., 1994; Zhang et al., 1996). Endoglin, also

Please address all correspondence to C. Bernabeu, Centro de Investigaciones Biologicas, CSIC, Velazquez 144, 28006 Madrid, Spain. Tel.: 34 1 5644562 ext. 4246. Fax: 34 1 5627518.

known as CD105, is a 180-kD homodimeric membrane glycoprotein expressed by human endothelial cells (Gougos and Letarte, 1988), macrophages (Lastres et al., 1992; O'Connell et al., 1992), erythroid precursors (Bühring et al., 1991), syncytiotrophoblast of term placenta (Gougos et al., 1992), and stromal cells (St Jacques et al., 1994; Rokhlin et al., 1995). The gene encoding endoglin has been localized to human chromosome 9 (Fernández-Ruiz et al., 1993) and has been identified recently as the target gene for the autosomal dominant vascular disorder known as hereditary haemorrhagic telangiectasia type 1 (MacAllister et al., 1994). Endoglin binds TGF-β1 and TGF-β3 with high affinity (k_D=50 pM) in human endothelial cells (Cheifetz et al., 1992). Two different isoforms, L-endoglin and S-endoglin, with the capacity to bind TGF-β, but differing in the amino acid composition of their cytoplasmic tails, have been characterized (Gougos and Letarte, 1990; Bellón et al., 1993). The predominant isoform L-endoglin contains 47 residues in the cytoplasmic domain, whereas S-endoglin has only 14 amino acids with the seven residues proximal to the transmembrane region being common to both isoforms. Interestingly, betaglycan and L-endoglin share a region of high identity in the cytoplasmic tail with a high content (40%) of potentially phosphorylated Ser/Thr residues (Gougos and Letarte, 1990; López-Casillas et al., 1991; Wang et al., 1991; Morén et al., 1992). In fact, human endoglin has been found to be constitutively phosphorylated in endothelial cells and mouse fibroblast transfectants expressing either L-endoglin or S-endoglin (Lastres et al., 1994). Endoglin is absent from peripheral monocytes, but it is expressed by in vitro differentiated monocytes and following phorbol ester treatment of monocytic cell lines (Lastres et al., 1992). Here, we have analyzed the potential role of endoglin in modulating TGF-β responses by transfecting cDNA encoding L- and S-endoglin into the U-937 monocytic line.

Materials and Methods

Cells and Stable Transfectants

Human promonocytic U-937 cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, penicillin (100 U/ml), and gentamycin (25 μg/ml) in a 5% CO_2 atmosphere at 37°C. Treatment of cells with recombinant human TGF-β1 (R&D Systems, Abingdon, UK) was performed at the concentrations and times indicated. Monocyte-derived macrophages were isolated by incubating peripheral blood mononuclear cells at 37°C, 5% CO_2 and 100% humidity in autologous plasma-coated plastic flasks (Lastres et al., 1992). Nonadherent cells were removed by washing with prewarmed Hank's solution. Adherent cells were harvested with Hank's medium containing 5 mM EDTA at 37°C for 5 min. The phenotype of the adherent population was >98% $CD11b^+$, $CD11c^+$, $CD31^+$, and <3% $CD3^+$, $CD2^+$.

U-937 transfectants expressing human L-endoglin or S-endoglin were generated by cotransfection of full-length cDNA encoding L-endoglin or S-endoglin in pcEXV and psV2neo at a ratio of 10:1, respectively (Bellón et al., 1993). Transfections were performed by electroporation of 3×10^7 cells in RPMI 1640 medium using a BTX 600 electroporator with 100 μg of plasmid DNA in 2-mm cuvettes. After selection in RPMI medium containing 10% FCS and 1.5 mg/ml of the antibiotic G418 (Sigma Chem. Co., St. Louis, MO), endoglin-positive cells were characterized by flow cytometry analysis. Parallel transfections with psV2neo alone yielded endoglin-negative mock transfectants. Pooled clones were used in biochemical and functional characterizations, except in quantitative PCR studies. No differences were observed between parental and mock transfectants in biochemical and functional studies.

Flow Cytometry

Cells (5×10^5) were incubated with specific mAb for 30 min at 4°C. After two washes with PBS, FITC-labeled $F(ab')_2$ rabbit anti-mouse Ig (Dakopatts) was added and incubation proceeded for an additional period of 30 min at 4°C. Finally, cells were washed twice with PBS and their fluorescence was estimated with an EPICS-CS (Coulter Científica, Móstoles, Spain), using logarithmic amplifiers. Antibodies used were mAb 8E11 (anti-endoglin) (Lastres et al., 1992), HC1/6 (anti-platelet/endothelial cell adhesion molecule 1 [PECAM-1][1]) (Cabañas et al., 1989), HP1/7 (anti-VLA-4), (Sánchez-Madrid et al., 1986), P1D6 (anti-VLA-5) (Telios Pharmaceuticals, Palo Alto, CA), and RR/1 (anti-intercellular adhesion molecule 1 [ICAM-1]) (generous gift of Dr. T. Springer, Center for Blood Research, Boston, MA).

Cell Surface Biotinylation

Cells were washed at 4°C with Hepes buffer (150 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Hepes, pH 7.4) and allowed to equilibrate for 30 min at 4°C in the same buffer. Cells were washed again and resuspended at 2×10^6 cells/ml in Hepes buffer containing 0.5 mg/ml of sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin, Pierce Chem. Co., Rockford, IL). After incubation at 4°C for 2 h, the reaction was stopped by washing twice with Hepes buffer. For immunoprecipitation studies, cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 50 μg/ml leupeptin, 1 mM benzamidine and 1 mM PMSF), for 40 min at 4°C. The lysates were centrifuged for 15 min at 12,000 *g* and the supernatants were precleared for 4 h with protein G coupled to Sepharose (Pharmacia Biotech, Barcelona, Spain) at 4°C. Specific immunoprecipitations of the precleared lysates were carried out in the presence of 10 μg/ml of the mAb 44G4 (anti-endoglin) (Gougos and Letarte, 1988), using protein G coupled to Sepharose. After overnight incubation at 4°C, immunoprecipitates were isolated by centrifugation and washed twice with lysis buffer at 4°C. Immune complexes were subjected to SDS-PAGE on a 7.5% acrylamide gel under nonreducing conditions and electrotransferred to nitrocellulose. Filters were blocked with 5% powder milk in PBS for 1 h and then incubated with 2 μg/ml of streptavidin conjugated to horseradish peroxidase (Pierce Chem. Co.) for 2 h at room temperature. Biotinylated endoglin was detected using an Enhanced ChemiLuminescence system (Amersham Ibérica S.A., Madrid, Spain).

RNA Preparation and Northern Blot Analysis

Total cellular RNA was isolated using guanidinium thiocyanate/phenol/chloroform (Chomczynski and Sacchi, 1987). RNA samples (10 μg) were denatured and then fractionated in 1.1% agarose/formaldehyde gels and blotted onto nitrocellulose. Membranes were hybridized in 50% formamide at 42°C with excess ^{32}P-labeled probes, washed under highly stringent conditions (0.2 × SSC and 0.5% SDS at 52°C), and radiolabeled bands were detected with a PhosphorImager 410A and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The probes used were the 2.2-kb EcoRI insert of endoglin in pcEXV-EndoS (Bellón et al., 1993) and the 1.5-kb ClaI–EcoRI fragment of pMA413 plasmid which contains the third exon of c-*myc* (Dalla Favera et al., 1982).

Analysis of TGF-β1 and Its Receptors by Quantitative PCR

U-937 transfectants were treated with or without 100 pM TGF-β1 for 72 h. Cells were washed and total cellular RNA was extracted (Chomczynski et al., 1987). Before reverse transcription the concentration of RNA was estimated in two independent determinations and at two different dilutions, as this is a crucial parameter in the quantitative analysis of mRNA expression (Murphy et al., 1990). The integrity of oligo(dT)-synthesized cDNA was confirmed by β-actin PCR. The oligonucleotides used for the amplification of actin, TGF-β1, TGF-β receptor II, endoglin and betaglycan, and the conditions for exponential amplification have been described previously (Jindal et al., 1995). Specific primers and an internal probe for TGF-β receptor I (ALK-5) were chosen from the published sequence (Franzén et al., 1993): primer A (5'-AATTCCTCGAGATAGGCCGT-3') correspond-

1. *Abbreviations used in this paper*: ICAM-1, intercellular adhesion molecule 1; PECAM-1, platelet/endothelial cell adhesion molecule 1.

ing to bp 307-327, primer B (5'-TGCGGTTGTGGCAGATATAG-3') corresponding to the complement of bp 530-511 and the internal probe (5'-TACAGTAGTTGGAAGTTCTA-3') complementary to the bp 421-402. Briefly, cDNA was serially diluted in water to concentrations ranging from 400 ng to 0.16 ng of corresponding RNA per sample and the PCR reaction (100 μl) performed in 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 0.5 μg each of 5'- and 3'- primers and 0.25 U of Taq DNA polymerase (Pharmacia, Montreal, Canada). The amplification profile involved preincubation at 98°C for 5 min, denaturation at 94°C for 75 s, primer annealing at 58°C for 75 s and extension at 72°C for 2 min. The PCR reaction is considered quantitative if an exponential range is obtained with each sample; this requires 28 cycles for β-actin, TGF-β1, R-I, R-II and endoglin, and 30 cycles for betaglycan (Jindal et al., 1995; Zhang et al., 1996). A 10-μl aliquot of PCR reaction mixture was electrophoresed in 4–20% gradient polyacrylamide gels in Tris-borate/EDTA buffer (Novel Experimental Technology, San Diego, CA). Gels were stained with ethidium bromide and photographed. Gels were also transferred to nylon membranes and hybridized with the specific internal probes 3'end-labeled with Digoxigenin-11-ddUTP; the DIG-labeled oligonucleotides were detected by chemiluminescence with Lumigen PPD according to manufacturer's instructions (Boehringer Mannheim, Biochemica, Montreal, Canada). Membranes were then exposed to Standard X-ray film for 2–5 min; each band was scanned on a Densitometer model 300A (Molecular Dynamics Co.). Background under each band was automatically subtracted and the densitometric units were recorded and plotted vs the corresponding concentration of RNA to ascertain the exponential range of the amplification as described previously (Jindal et al., 1995; Zhang et al., 1996).

Proliferation Assays

U-937 cells were cultured in flat bottomed 96-well plates (Costar, Cambridge, MA) at 3×10^4 cells/well in RPMI with 10% FCS in the absence or in the presence of TGF-β1 for the times indicated, the last 6 h in the presence of 1 μCi per well of (^{3}H-methyl)-thymidine (^{3}H-TdR, Amersham, UK). Cells from triplicate samples were collected using a harvester (Skatron, Norway) and ^{3}H-Thy incorporation into DNA was measured in a liquid scintillation counter.

Western Blot Analysis

U-937 cells were cultured at a density of 5×10^5 cells/ml in RPMI containing 10% FCS. In some experiments, cells were treated with either 500 pM TGF-β for 72 h or 10 ng/ml of PMA for 48 h. Cells were collected by centrifugation, lysed with lysis buffer and extracts corresponding to 2×10^6 cells were subjected to SDS-PAGE on 7.5% polyacrylamide gels under nonreducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA). Filters were blocked with PBS containing 5% milk powder for 1 h. Specific immunodetection was carried out by incubation with mAb 44G4 (anti-endoglin) overnight, followed by peroxidase-conjugated goat anti–mouse Ig at room temperature. The presence of endoglin was revealed using a chemiluminescence assay (ECL detection kit, Amersham Ibérica). Quantitative estimations were performed by densitometry of the autoradiograms using the ImageQuant software (Molecular Dynamics).

Metabolic Labeling of PECAM-1

U-937 cells were incubated with or without 500 pM of TGF-β1 for 20 h. Then, cells were preincubated for 30 min at 37°C in phosphate-free or methionine/cysteine-free RPMI medium containing 10% dyalized FCS. Medium was removed, fresh medium containing 100 μCi/ml of [^{32}P]orthophosphate or 50 μCi/ml of [^{35}S]methionine/cysteine (ICN Biomedicals, Inc., Costa Mesa, CA) was added and the cells were metabolically labeled at 37°C in a 5% CO_2 atmosphere for 4 h with or without 500 pM of TGF-β1. Cells were then collected by centrifugation, washed twice at 4°C with PBS, and lysed in 250 μl of lysis buffer containing a phosphatase inhibitor cocktail (1 mM orthovanadate, 1 mM sodium molybdate, and 1 mM sodium fluoride). The lysates were centrifuged for 15 min at 12,000 *g* and the supernatants were precleared for 4 h with protein G–Sepharose (Pharmacia Biotech) at 4°C. Specific immunoprecipitations of the precleared lysates were carried out in the presence of mAb HC1/6 (anti–PECAM-1), using protein G–Sepharose. After overnight incubation at 4°C, immunoprecipitates were isolated by centrifugation, washed twice with lysis buffer at 4°C, and then subjected to SDS-PAGE on a 7.5% acrylamide gel under nonreducing conditions. Detection of the ^{32}P-labeled PECAM-1 was revealed with a PhosphorImager 410A and ImageQuant software (Molecular Dynamics). Detection of ^{35}S-labeled bands was revealed with standard autoradiography (Kodak).

Cellular Aggregation Assays

U-937 transfectants were cultured in flat-bottom 96-well plates (Costar) at 3×10^4 cells/well and incubated either in the absence or in the presence of 500 pM of TGF-β1 for 24 h in RPMI containing 10% FCS at 37°C and 5% CO_2 in triplicate wells. Cell aggregates formation was visualized on the plate with a phase contrast microscope (Nikon Diaphot, Tokyo, Japan).

Cellular Adhesion Assays

Adhesion to uncoated surfaces was performed by incubating U-937 transfectants (5×10^4 cells/well) either in the absence or in the presence of 500 pM of TGF-β1. For cellular adhesion to fibronectin-coated surfaces, 96-well plates were coated with 1 μg/cm^2 of human fibronectin in 100 μl (Sigma Chem. Co.) at 37°C for 1 h. Wells were washed three times with PBS and incubated with 1% BSA in PBS at 37°C for an additional period of 1 h; then, wells were rinsed three times with PBS. Cells treated with TGF-β1 at 500 pM for 24 h were washed, resuspended in RPMI containing 1% BSA and 10 mM Hepes at a concentration of 5×10^5 cells/ml, and 100 μl of the cell suspension were added to each well. Incubations were carried out for 1 h at 37°C in triplicate samples.

At the times indicated (see figure legends for details), culture medium was removed and the wells gently washed twice with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells attached were fixed overnight with 1% glutaraldehyde in PBS at room temperature. Plates were rinsed three times and cells stained with 0.1% toluidine blue during 2 h at room temperature. Additionally, wells were rinsed three times with PBS and cell associated dye was extracted with 100 μl of 10% acetic acid. The optical density in the wells was measured at 628 nm with a Multiscan Biochromatic (Labsystem, Helsinki, Finland). Percentage of cells attached was calculated using a calibration curve.

Fibronectin Production

U-937 transfectants were incubated in the presence of 500 pM TGF-β1 in RPMI 10% FCS at 37°C under 5% CO_2 atmosphere for the times indicated. Metabolic labeling was carried out for the last 4 h of treatment in 2 ml of serum-free medium (ICN Biomedicals, Barcelona, Spain) containing 50 μCi/ml of [^{35}S]methionine/cysteine (Trans-^{35}S label, ICN Biomedicals) at a density of 2.5×10^6 cells/ml. Culture supernatants were collected, centrifuged at 12,000 *g* for 5 min and precleared for 4 h with Sepharose in the presence of 0.1% Triton X-100 and 1 mM PMSF. Specific precipitation of soluble fibronectin was carried out with 50 μl of packed gelatin-Sepharose (Pharmacia Biotech) at 4°C overnight. Precipitates were isolated by centrifugation, washed twice with PBS at 4°C and subjected to SDS-PAGE on a 6% acrylamide gel under reducing conditions. Detection of radiolabeled bands was revealed with standard autoradiography (Kodak).

Specific Binding of ^{125}I-TGF-β1

U-937 cells expressing endoglin and control mock transfectants were washed in PBS and equilibrated for 40 min in RPMI 1640 containing 25 mM Hepes buffer, pH 7.0, and 2 mg/ml BSA. Between 4 and 7×10^6 cells/sample were incubated for 4 h at 4°C with 25–250 pM of ^{125}I-TGF-β1 with or without a 40-fold excess of competing unlabeled TGF-β1 and in a final volume of 1.0 ml in siliconized Eppendorf tubes. ^{125}I-TGF-β1 (800–2,000 Ci/mmol) was either kindly provided by Celtrix Pharmaceuticals (Santa Clara, CA) or purchased from Amersham Ibérica. Cells were washed twice in a 0.05-M Hepes buffer, pH 7.5, containing 128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$ (buffer A) supplemented with 2 mg/ml BSA, and washed once and resuspended in 1.0 ml of buffer A, essentially as described previously (Massagué, 1987). Cell-associated radioactivity was estimated in a gamma counter.

Receptor Affinity Labeling

Affinity labeling assays were basically performed as described (Massagué, 1987). Briefly, U-937 cells expressing S-endoglin or L-endoglin and mock transfectants were incubated in Hepes buffer containing 0.1% BSA with 50–250 pM of ^{125}I-TGF-β1 for 4 h with or without a 50-fold excess of com-

peting unlabeled TGF-β1. Cells were washed and radiolabeled TGF-β1 was cross-linked with 0.30 mM disuccinimidyl suberate (Pierce Chemical Co.) in Hepes buffer for 15 min at 4°C. Cells were washed four times and solubilized in lysis buffer. The total extracts were directly subjected to SDS-PAGE analysis or to specific immunoprecipitations with mAb 44G4 (anti-endoglin). Detection of the ^{125}I-labeled receptors was revealed using a PhosphorImager 410A (Molecular Dynamics).

Degradation of ^{125}I-TGF-β1 Assays

Degradation assays were basically performed as described (Frolik et al., 1984). Briefly, U-937 transfectant cells were incubated at 37°C for 1 h in the presence of 100 pM of ^{125}I-TGF-β1 at 5 × 10^6 cells/ml in DMEM medium containing 0.1%BSA, 25 mM Hepes, pH 7.4. Cells were washed extensively with fresh medium and triplicate aliquots (2.5 × 10^6 cells) were transferred to new tubes and incubated for an additional period of 30 min, 1 h or 2 h at 37°C. As a control, some aliquots were maintained on ice (time = 0). At the end of each time interval, cells were pelleted by centrifugation at 700 *g* for 3 min at 4°C and the supernatants were removed. Aliquots of 500 μl were precipitated with 10% TCA on ice. Soluble and insoluble material were separated by centrifugation at 12,000 *g* at 4°C for 10 min. For each incubation time, cell-associated radioactivity, total radioactivity in supernatants and TCA soluble radioactivity were estimated in a gamma counter (LKB, Bromma, Sweden). Total radioactivity present in the samples (cell associated plus supernatant) was considered as 100%.

Results

TGF-β Upregulates Endoglin Expression in Monocytic Cells

We have previously reported that endoglin is upregulated during monocyte differentiation (Lastres et al., 1992). Since TGF-β is able to synergize with other factors in macrophage differentiation (De Benedetti et al., 1990; Testa et

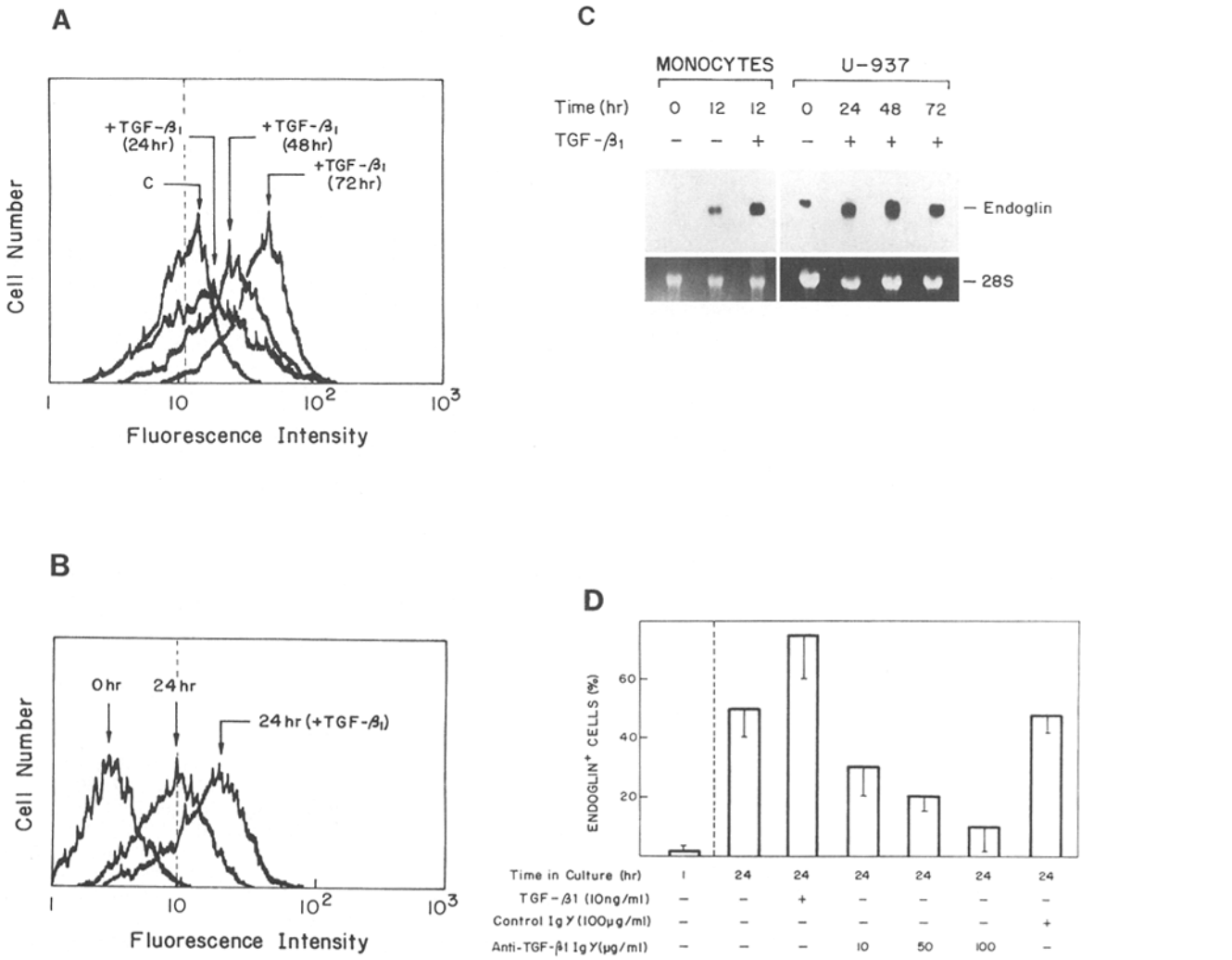


Figure 1. Effect of TGF-β on the expression of endoglin by monocytic cells. (*A*) U-937 cells were incubated in the absence (*C*) or in the presence of 500 pM TGF-β1 for 24, 48, and 72 h, respectively. The reactivity of 8E11 (anti-endoglin) mAb was assayed by flow cytometry. The broken vertical line represents the gate determined in the control staining with an irrelevant mAb. (*B*) Human peripheral monocytes freshly harvested or incubated with or without 500 pM TGF-β1 for 24 h were analyzed for endoglin expression by flow cytometry as described in *A*. (*C*) RNA was prepared from monocytes and U-937 cells incubated with or without 500 pM TGF-β1 for the times indicated. Endoglin transcripts of 3.4 kb were detected by Northern blot analysis. The blots were also stained by ethidium bromide to visualize the 28S ribosomal RNA. (*D*) Peripheral monocytes were cultured in the presence of a neutralizing antibody to TGF-β, a control antibody, or exogenous TGF-β. At the times indicated, the expression of endoglin was estimated by flow cytometry using an anti-endoglin mAb. Similar results were obtained when using a serum-free medium. The mean of three different experiments ± SD is shown.

al., 1993), and it is chemotactic for monocytic cells (Wahl et al., 1987), we wondered whether TGF-β would affect expression of endoglin. Low levels of endoglin were detected on the surface of U-937 cells; however, when U-937 cells were cultured in the presence of TGF-β endoglin expression was clearly increased up to 3–4-fold after 72 h of treatment (Fig. 1 *A*). Addition of exogenous TGF-β to peripheral blood monocytes was also able to increase by 4–5-fold the expression of endoglin after 24 h in culture (Fig. 1 *B*). This regulatory effect was evident not only at the cell surface, but also when the levels of specific mRNA transcripts were analyzed in both monocytes and U-937

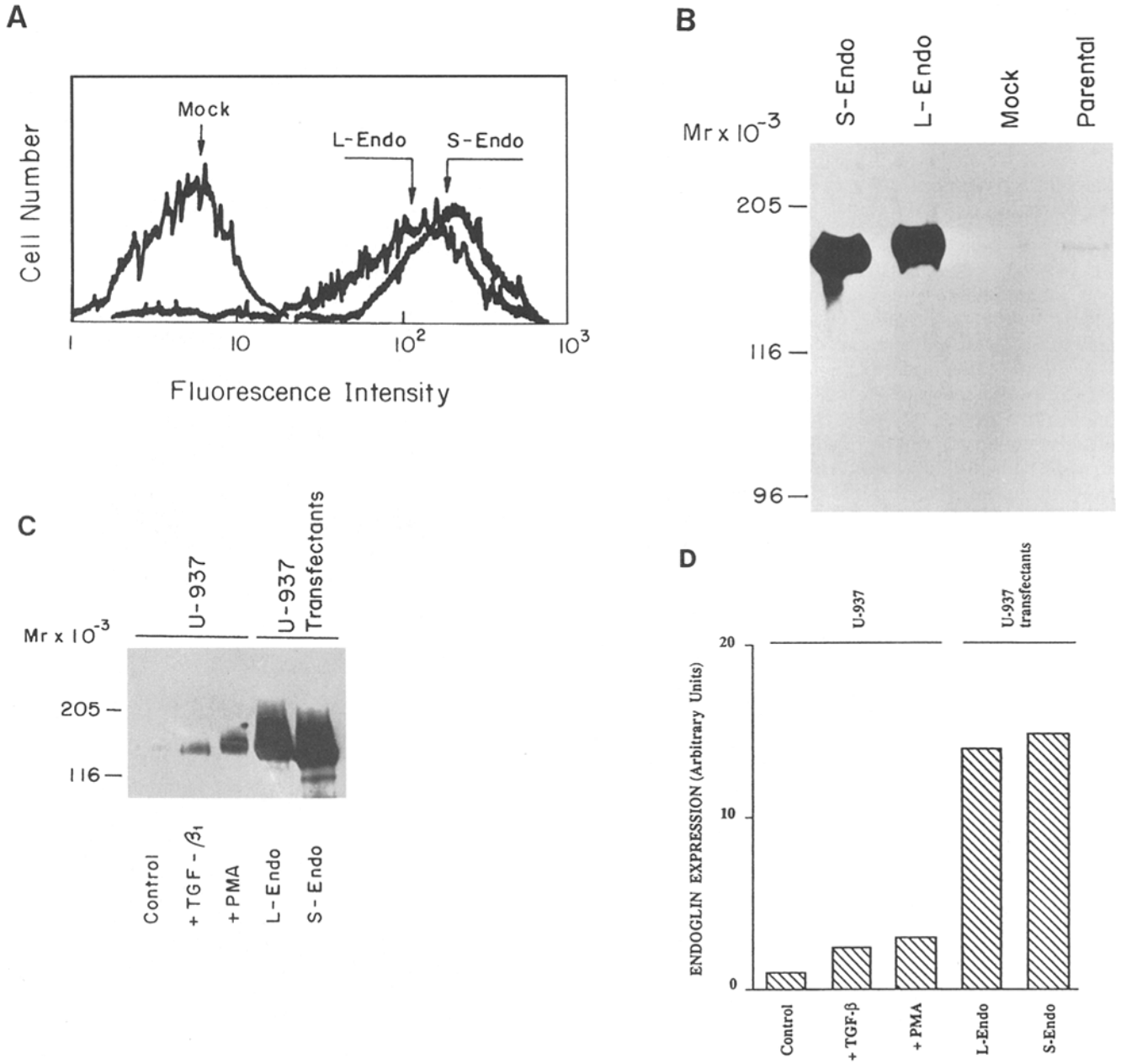


Figure 2. Characterization of U-937 endoglin transfectants. U-937 cells were transfected with L- or S-endoglin cDNA or with vector alone (*Mock*) and the expression of the endoglin molecule analyzed. (*A*) Analysis by cytofluorometry of the endoglin present at the cell surface. Cells were stained by indirect immunofluorescence with an anti-endoglin mAb. The mean fluorescence intensities of mock, L-endoglin, and S-endoglin transfectants were 7, 163, and 191, respectively. (*B*) Immunoprecipitation analysis. Cells were surface labeled with biotin, lysed, and immunoprecipitated with anti-endoglin mAb. Samples were electrophoresed on a 7.5% acrylamide gel under nonreducing conditions, transferred to nitrocellulose, and the biotinylated endoglin detected using a chemiluminescence assay. (*C*) Western blot analysis. Extracts from untreated, TGF-β1–treated, or PMA-treated parental U-937 cells and L-endoglin or S-endoglin transfectants were electrophoresed and transferred to nitrocellulose membranes. The presence of endoglin was revealed with anti-endoglin mAb using a chemiluminescence assay. (*D*) The autoradiogram shown in *C* was subjected to densitometric analysis using the ImageQuant software. Relative levels of endoglin in parental U-937 cells and L-endoglin (*L-Endo*) or S-endoglin (*S-Endo*) transfectants are shown.

cells (Fig. 1 *C*). Furthermore, TGF-β appears to be capable of mediating the upregulation of endoglin observed on monocytes in culture (Lastres et al., 1992) as this was prevented by the addition of a neutralizing polyclonal antibody to TGF-β1 (Fig. 1 *D*). Similar results were obtained when peripheral blood monocytes were cultured in serum-free medium (data not shown), suggesting that endogenous production and/or activation of TGF-β by the monocytes might be responsible for the upregulation of endoglin. Since macrophages are able to synthesize this ligand (Assoian et al., 1987), it is tempting to speculate the involvement of autocrine TGF-β1 in the upregulation of its own receptor. These results demonstrate that TGF-β induces the expression of endoglin in monocytic cells.

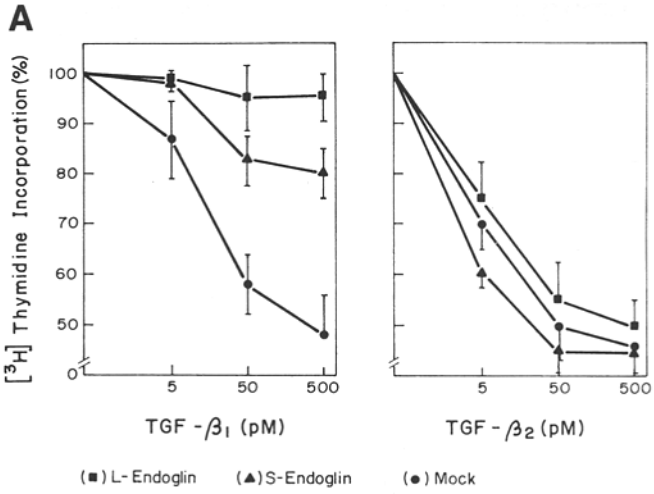
Endoglin Expression Interferes with Cellular Responses to TGF-β

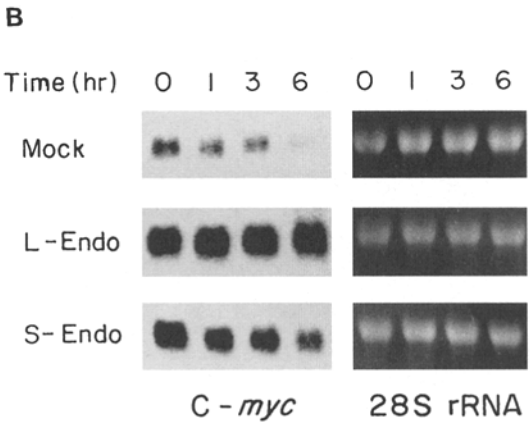
To study the role of endoglin in TGF-β signaling, L-endoglin and S-endoglin transfectants were generated in the U-937 human monocytic line. Fig. 2 *A* demonstrates that both forms of endoglin are expressed at high levels on the surface of U-937 transfectants, as determined by flow cytometry analysis. Mean fluorescence intensity values indicated that the level of endoglin expressed on L- and S-endoglin transfectants were 23–27 times that of mock or parental cells. Immunoprecipitation analysis revealed a 170-kD dimer (L-endoglin) or a 160-kD dimer (S-endoglin) specifically recognized by mAb 44G4 (Fig. 2 *B*). The level of recombinant endoglin expressed in the transfectants was also analyzed by Western blot (Fig. 2 *C*) and found to be significantly higher than that induced in U-937 cells treated with TGF-β or phorbol esters (3–4-fold) (Fig. 2 *D*). Differences observed in the levels of endoglin expressed by transfectants relative to control when estimated by flow cytometry (23-27) or Western blot (13-16) analyses, might be explained by methodological differences between these immunodetection techniques. Several biological responses of monocytes to TGF-β, which had been described previously, were analyzed in the endoglin transfectants and compared to that of parental or mock-transfected lines.

Cellular Proliferation and c-myc Regulation

As demonstrated previously, 24 h incubation with TGF-β1 or -β2 inhibit the proliferation of U-937 cells in a dose-dependent manner (Lastres et al., 1994; Fig. 3 *A*). However, L-endoglin transfectants were not inhibited at all by TGF-β1 while S-endoglin transfectants were only partially inhibited (Fig. 3 *A*). The decrease in proliferation induced by TGF-β2 was not altered in the endoglin transfectants, which is in agreement with the known binding specificity of endoglin for the β1 isoform and not the β2 isoform (Cheifetz et al., 1992).

In U-937 cells, a decrease in c-*myc* transcript levels is associated with inhibition of proliferation induced by TGF-β1 (Lastres et al., 1994). Fig. 3, *B* and *C* show a substantial





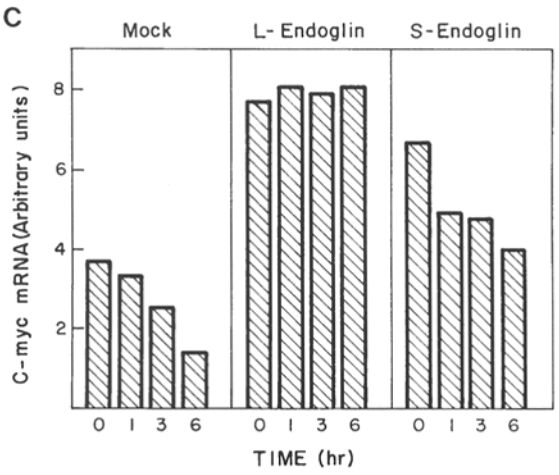


Figure 3. Effects of TGF-β1 on cellular proliferation and expression of c-*myc* transcripts in U-937 endoglin transfectants. (*A*) Transfectants were incubated with either TGF-β1 or TGF-β2 at different concentrations. The proliferative capacity of the cells was measured after 24 h in culture by [^{3}H]thymidine incorporation. The absolute cpm of untreated mock, L-endoglin and S-endoglin transfectants were 55,307 ± 5,906, 50,132 ± 4,546, and 60,333 ± 3,399, respectively. The mean ± SD of three different experiments performed in triplicate samples is shown. (*B*) Effect of endoglin expression on the c-*myc* levels detected in the presence of 500 pM TGF-β1. Transfectants were incubated with or without 500 pM TGF-β1 for the times indicated and the levels of specific transcripts of c-*myc* mRNA detected by Northern blot analysis. RNA blots were stained with ethidium bromide to visualize the 28S rRNA. The levels of c-*myc* mRNA were quantitated by densitometric analysis using the ImageQuant software and are illustrated in *C*.

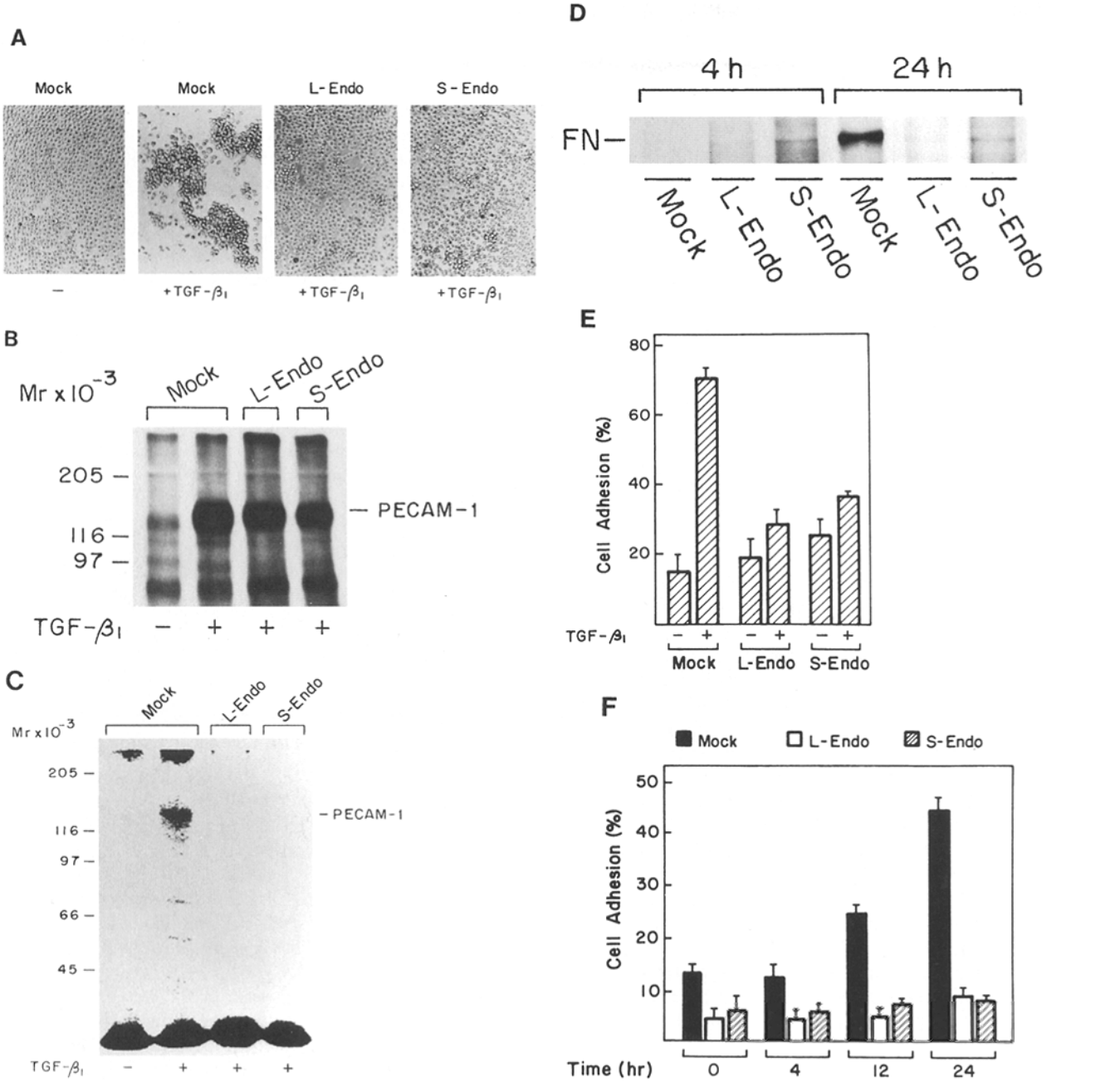


Figure 4. Effect of TGF-β1 on cell adhesion receptors and fibronectin synthesis of U-937 endoglin transfectants. (*A*) Effect of endoglin expression on the TGF-β1–induced homotypic aggregations. Cell transfectants were incubated either in the absence or in the presence of 500 pM TGF-β1 for 24 h and aggregate formation was visualized by phase contrast microscopy. (*B*) Immunoprecipitation analysis of PECAM-1. Cell transfectants were incubated either in the absence or in the presence of 500 pM TGF-β1 for 20 h, metabolically labeled with [^{35}S]methionine/cysteine and lysed as described in Materials and Methods. Lysates were immunoprecipitated with mAb HC1/6 to PECAM-1 and protein G–Sepharose. Samples were subjected to SDS-PAGE and standard autoradiography. (*C*) Effect of endoglin expression on the TGF-β1 dependent phosphorylation of PECAM-1. Cell transfectants were incubated either in the absence or in the presence of 500 pM TGF-β1 for 20 h, metabolically labeled with [^{32}P]orthophosphate and lysed as described in Materials and Methods. Lysates were immunoprecipitated with protein G–Sepharose in the presence of the mAb HC1/6 to PECAM-1. Samples were subjected to SDS-PAGE followed by detection of radiolabeled bands on the gel with the use of a PhosphorImager 410. (*D*) Effect of endoglin expression on the TGF-β1–induced fibronectin synthesis. Cell transfectants were incubated in the presence of 500 pM TGF-β1 for the times indicated and metabolically labeled with [^{35}S]methionine/cysteine for 4 h. Soluble fibronectin was precipitated from the supernatants using gelatin-Sepharose. Samples were subjected to SDS-PAGE followed by detection of radiolabeled bands by autoradiography. (*E*) Effect of endoglin expression on the TGF-β1–induced cellular adhesion to fibronectin-coated surfaces. U-937 transfectants were incubated either in the absence or in the presence of 500 pM of TGF-β1 in RPMI containing 10% FCS. After 24 h in culture, cells were washed, resuspended in RPMI containing 1% BSA and 10 mM Hepes, and incubated in fibronectin-coated wells. After 1 h, culture medium was removed and the wells gently washed twice. Cells attached were fixed overnight with 1% glutaraldehyde and stained with 0.1% toluidine blue. Cell-associated dye was extracted with 10% acetic acid and the optical density in the wells was measured at 628 nm with a Multiscan Biochromatic. The mean of triplicate samples ± SD is shown. (*F*) Effect of endoglin expression on the TGF-β1–induced cellular adhesion to uncoated surfaces. U-937 transfectants were incubated either in the absence or in the presence of 500 pM of TGF-β1 on uncoated 96-well plates. At the times of treatment indicated, culture medium was removed and the wells gently washed twice. The number of cells attached were estimated as described in *C*.

downregulation of these transcripts in mock transfectants 6 h after addition of TGF-β1. In the L-endoglin transfectants, a 2–3-fold increase in the basal level of c-*myc* transcripts was observed and these levels were unaffected by the addition of ligand. The S-endoglin transfectants also showed higher basal levels of c-*myc* transcripts; however, a decrease in these levels could be induced by treatment with TGF-β1. Thus, S-endoglin transfectants show an intermediary response in terms of both c-*myc* downregulation and inhibition of cellular proliferation. However, the presence of L-endoglin in U-937 cells totally suppressed c-*myc* downregulation and inhibition of proliferation. Taken together, these results suggest that endoglin expression interferes with the inhibition of cellular proliferation induced by TGF-β1.

PECAM-1 Upregulated Expression, Phosphorylation, and Homotypic Aggregation

We have previously demonstrated that TGF-β1 induces the expression of PECAM-1 in U-937 cells and its activation by phosphorylation which in turn induces the homotypic aggregations of these cells (Lastres et al., 1994). In the current study, we noted that TGF-β1 was unable to induce the aggregation of the endoglin transfectants while it stimulated that of the parental cells or mock transfectants (Fig. 4 *A*). We thus assessed whether the inability to form cellular aggregates was due to a lack of induction of PECAM-1 or to an inability to phosphorylate this molecule. We observed a 2–3-fold increase in the level of PECAM-1 following treatment of parental U-937 cells with TGF-β1 as measured by flow cytometry analysis (Lastres et al., 1994). A similar level of induction was seen with the mock transfectants, and the S- and L-endoglin transfectants which showed a 2.7-, 3.3-, and 2.6-fold increase of PECAM-1 expression, respectively (Table I). Fig. 4 *B* confirms that the presence of endoglin does not alter the ability of the cells to upregulate PECAM-1 revealed as a protein of M_r = 130,000 by immunoprecipitation of ^{35}S-labeled cells. However, no phosphorylated PECAM-1 could be detected by immunoprecipitation with specific antibodies from L- or S-endoglin transfectants, whereas it could be readily observed in mock transfectants (Fig. 4 *C*). Thus, although the presence of endoglin does not impair the upregulation of PECAM-1 in response to TGF-β1, it prevents its phosphorylation and the induction of homotypic aggregation.

Induction of Fibronectin Synthesis

In several cell types, TGF-β is capable of inducing the synthesis of fibronectin (Massagué, 1990). We thus assessed the ability of U-937 cells and their endoglin transfectants to synthesize this extracellular matrix component. Fig. 4 *D* illustrates that fibronectin synthesis is induced by TGF-β1 in mock transfectants, particularly after 24 h of treatment. By contrast, transfectants expressing either L- or S-endoglin were less responsive to ligand. Thus, endoglin interferes with the ability of U-937 cells to synthesize fibronectin in response to TGF-β1.

Cell Adhesion Receptors

It has been observed previously that treatment with TGF-β1 enhances the attachment of U-937 cells to fibronectin through the $\alpha_5\beta_1$ integrin receptor (Bauvois et al., 1992). Thus, we wondered whether binding of these receptors to a fibronectin-coated surface was affected in endoglin transfectants. Mock transfectants showed a marked increase in cellular adhesion capacity, while endoglin transfectants showed a much reduced stimulation of adhesion by TGF-β1 (Fig. 4 *E*). In addition, the ability of endoglin transfectants to adhere to uncoated plates was totally inhibited, whereas mock transfectants showed a time-dependent increase in adhesion (Fig. 4 *F*). These results suggest that membrane receptors for fibronectin and possibly for other extracellular matrix components are affected in endoglin transfectants.

TGF-β1 has been shown to upregulate the synthesis of $\alpha_5\beta_1$ integrin and to downregulate the expression of $\alpha_4\beta_1$ and of intercellular adhesion molecule 1 (ICAM-1) in monocytic cells (Bauvois et al., 1992; Wahl et al., 1993; Lastres et al., 1994). We confirmed these results here and noted no difference between endoglin and mock transfectants in the modulation of their integrins in response to TGF-β1. A 1.5–1.7-fold increase in $\alpha_5\beta_1$ integrin was observed compared to a 1.3–1.7-fold decrease in $\alpha_4\beta_1$ integrin and ICAM-1 in all groups following treatment with ligand (Table I). Thus, modulation of the levels of adhesion receptors was not influenced by the presence of endoglin. However, activation of $\alpha_5\beta_1$ integrin, which is necessary for adhesion to fibronectin, might not occur in the endoglin transfectants.

TGF-β1 Upregulates Itself and Its Receptors in U-937 Cells and Endoglin Overexpression Does Not Alter the Response

To determine whether the upregulation of endoglin observed in Fig. 1 could be extended to other TGF-β receptors, we performed quantitative RT-PCR analysis on U-937 cells treated with or without 100 pM TGF-β1 for 72 h. To facilitate the comparison of receptors levels between the endoglin transfectants and mock or parental groups, relative levels of RNA were estimated by quantitative β-actin RT-PCR. The 318-bp PCR product that specifically hybridized to the β-actin probe was estimated at different concentrations of corresponding RNA and the exponential range of amplification determined. Fig. 5 *A* illustrates the levels of β-actin mRNA at a concentration within this exponential range (0.5 ng/sample). In all experiments, the exponential range of detection of each of the receptors was determined and corrections made for the relative levels of RNA in these samples. Fig. 5 *A* shows a

Table I. Effect of TGF-β on the Expression of Cell Adhesion Receptors

	Mock		L-Endoglin		S-Endoglin	
TGF-β	−	+	−	+	−	+
PECAM-1	22 (100)	60 (273)	20 (100)	52 (260)	18 (100)	60 (333)
$\alpha_4\beta_1$	25 (100)	15 (60)	22 (100)	16 (73)	20 (100)	16 (80)
$\alpha_5\beta_1$	18 (100)	31 (172)	17 (100)	27 (159)	17 (100)	26 (153)
ICAM-1	17 (100)	13 (76)	13 (100)	10 (77)	16 (100)	10 (63)

U-937 transfectants were treated with or without 500 pM TGF-β for 24 h and stained with mAb to cell adhesion receptors. Values represent the mean channel fluorescence intensity obtained by flow cytometry. Expression levels relative to that of untreated transfectants (arbitrary value = 100) are shown in parentheses.

representative experiment for each of the receptors and Fig. 5 *B* demonstrates the exponential range reached and how the stimulation of receptor expression was determined.

Receptor I mRNA expression, as estimated in the exponential range of amplification of a specific 224-bp product, was stimulated ninefold by treatment of U-937 cells with TGF-β1. Receptor II mRNA level measured as a 261-bp product was upregulated 14-fold, while the 364-bp specific betaglycan PCR product was stimulated fourfold (Fig. 5, *A* and *B*). Thus, TGF-β1 can upregulate not only endoglin, but also R-I, R-II, and betaglycan in U-937 cells.

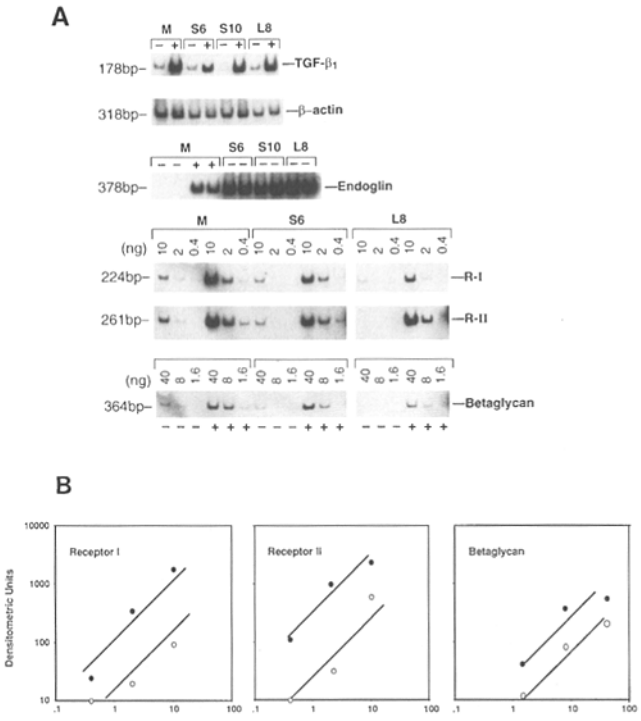


Figure 5. TGF-β1 upregulates itself and its receptors and the presence of endoglin does not alter these responses. (*A*) Mock (*M*), short endoglin (*S6* and *S10* clones), and long-endoglin (*L8*) U-937 transfectants were incubated with (+) or without (−) 100 pM TGF-β1 for 72 h at 37°C. RNA was extracted and RT-PCR was carried out to analyze the expression of TGF-β1 and endoglin using 10 ng/lane or at the concentrations indicated for R-I, R-II, and betaglycan. Quantitation of β-actin mRNA was performed on each sample by RT-PCR to estimate the relative amount of RNA present. The single concentration shown here (0.5 ng RNA/sample) was within the exponential range. 10 μl of each PCR mixture was fractionated by electrophoresis, transferred, hybridized with digoxigenin-labeled probes, and specific bands detected by chemiluminescence. Expression of R-I, R-II, and betaglycan mRNA was analyzed at three different concentrations and is illustrated for Mock, S6, and L8 transfectants. (*B*) Example of the quantitative measurement by RT-PCR of the increase in TGF-β receptors expression observed after treatment of cells with (●) or without (○) 100 pM TGF-β1 is shown for the mock transfectants. After RT-PCR and hybridization with the specific probes as shown in *A*, each band was scanned and the densitometric units were plotted vs the corresponding RNA concentration. The lines drawn represent the exponential range of amplification and the increase in mRNA levels is calculated within this range for R-I, R-II, and betaglycan.

As it was reported previously that TGF-β1 could upregulate its own expression in macrophages (Assoian et al., 1987; McCartney-Francis et al., 1990), we also analyzed whether it could also do so in U-937 cells. Fig. 5 *A* illustrates that the specific 178-bp TGF-β1 PCR product is increased substantially after TGF-β1 treatment. We then analyzed whether transfection of endoglin in U-937 cells could alter the upregulation of TGF-β1 and its receptors. Fig. 5 *A* demonstrates that mock transfectants, two independent clones of S-endoglin (S6 and S10) and one clone of L-endoglin (L8) transfectants, all responded similarly to TGF-β1 treatment. Data were analyzed similarly to those shown in Fig. 5 *A* and revealed no significant alteration by transfected endoglin of the stimulation of mRNA levels for TGF-β1 and its receptors in U-937 cells. Unfortunately, the possible TGF-β1 mediated endoglin upregulation in endoglin transfectants could not be determined due to the interference of the elevated background levels of recombinant protein and mRNA.

Effect of Endoglin Expression on TGF-β Binding and Degradation

Given the modulatory effect of endoglin expression on the TGF-β signaling, it was of interest to analyze whether ligand binding to the specific receptors or degradation of TGF-β were affected. First, TGF-β1 binding to cell transfectants was carried out at different concentrations of ligand. Fig. 6 *A* shows that specific binding to mock and L-endoglin transfectants was similarly increased in a dose-dependent manner, whereas binding to S-endoglin expressing cells was slightly increased (up to 150% at 250 pM), with respect to mock cells. This was confirmed in four different experiments. Next, the identification of the receptors bound to TGF-β1 in the U-937 transfectants was measured by affinity labeling and chemical cross-linking at three different concentrations of ligand (50, 150, and 250 pM). SDS-PAGE analysis of total lysates revealed a major 70-kD band of the putative R-I, and additional bands of 100–120 kD (likely R-II), 190–200 kD (endoglin), and high molecular weight oligomers (Fig. 6 *B*). As a control, L-endoglin (200 kD) and S-endoglin (190 kD) could be specifically immunoprecipitated from transfectants. Interestingly, the putative R-I displayed a significant increase of ligand binding in endoglin transfectants with respect to mock transfectants and it was coimmunoprecipitated with endoglin. Overall, these results did not reveal a decreased binding to the signaling receptors and rule out that the loss of certain TGF-β responses in endoglin transfectants was due to deficient ligand binding. Finally, cell associated and degraded (TCA soluble) radiolabeled TGF-β were determined at different times of incubation at 37°C. As shown in Fig. 6 *B*, no significant differences were found between mock and endoglin transfectants in either the cell associated or degraded TGF-β1, suggesting that the TGF-β degradation rate of endoglin transfectants cannot account for the loss of TGF-β responses. Further studies are needed to understand the mechanism of action of endoglin.

Discussion

The TGF-β receptor system is composed of several membrane proteins which include the receptors type I and II,

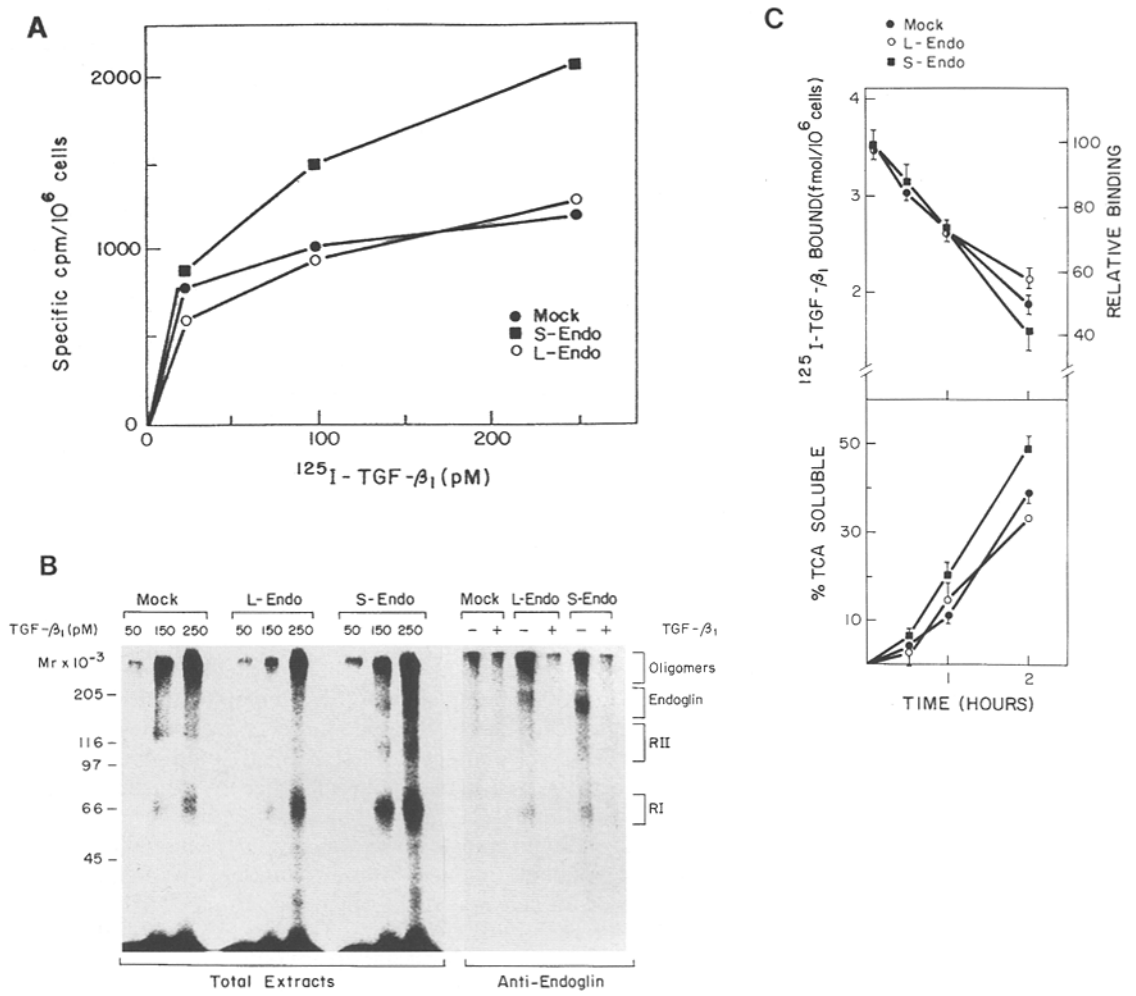


Figure 6. Effect of endoglin expression on TGF-β1 binding and degradation. (*A*) Binding of ^{125}I-TGF-β1 to U-937 transfectants. U-937 transfectants (7×10^6 cells/sample) expressing S-endoglin (*S-Endo*), L-endoglin (*L-Endo*), and mock transfectants (*Mock*), were incubated for 4 h at 4°C with 25–250 pM ^{125}I-TGF-β1 and washed as described in Materials and Methods. The nonspecific binding observed in the presence of a 40-fold excess unlabeled TGF-β1 was subtracted from the total cpm bound, to calculate the specific binding, and expressed as cpm bound per 10^6 cells. (*B*) Identification of the TGF-β1 receptors present on U-937 endoglin transfectants. U-937 transfectants expressing L-endoglin (*L-Endo*) or S-endoglin (*S-Endo*) and mock transfectants (*Mock*) cells were affinity labeled by incubation with 50, 150, or 250 pM of ^{125}I-TGF-β1, followed by chemical cross-linking with disuccinimidyl suberate. Cell extracts corresponding to the incubation at 250 pM ^{125}I-TGF-β1 either in the absence (−) or in the presence (+) of a 50-fold excess unlabeled TGF-β1, were immunoprecipitated with anti-endoglin mAb. Total extracts and immunoprecipitates were analyzed by SDS-PAGE on a 7.5% acrylamide gel under nonreducing conditions. Radiolabeled receptors were detected on the dried gel with a PhosphorImager 410A. The positions of L-endoglin (200 kD), S-endoglin, (190 kD), putative R-I (70 kD) and R-II (100–120 kD), and oligomers (>200 kD) are indicated. (*C*) Degradation of ^{125}I-TGF-β1 bound to U-937 transfectants. U-937 transfectants expressing S-endoglin (*S-Endo*), L-endoglin (*L-Endo*), and mock transfectants (*Mock*) were incubated for 1 h at 37°C in the presence of 100 pM ^{125}I-TGF-β1. After affinity labeling, cells were washed with fresh medium and triplicate aliquots of cells (2.5×10^6 each) were reincubated at 37°C for the time periods indicated. At the end of each time interval, cells were pelleted and the supernatants were collected. Supernatant aliquots were precipitated with 10% TCA on ice. Cell-associated radioactivity, total radioactivity in supernatants, and TCA soluble radioactivity were estimated in a gamma counter. Percentages were calculated as described in Materials and Methods. The mean of triplicate samples ± SD is shown.

betaglycan, and endoglin. The heteromeric association of the serine/threonine kinase receptors I and II, brought about by ligand binding, and the phosphorylation of R-I by R-II have been described as important elements in mediating TGF-β responses (Franzen et al., 1993; Bassing et al., 1994; Wrana et al., 1994; Laiho et al., 1991; Lin et al., 1992; Wrana et al., 1992; Cárcamo et al., 1994; Koenig et al., 1994). Betaglycan has been shown previously to potentiate the binding of ligand to cells and both betaglycan and endoglin have been suggested to associate with the signaling complexes (López-Casillas et al., 1993; Moustakas et al., 1993; Yamashita et al., 1994). In the current study, we demonstrate that endoglin when transfected in U-937 cells, can alter specific responses of cells to TGF-β1, suggesting that it can modulate the signals transmitted by the receptor complex.

That endoglin is a critical component of endothelial cells was demonstrated recently by the observation that mutations in the coding region of endoglin lead to a vascular disorder known as hereditary haemorrhagic telangiectasia

characterized by repeated and abundant nose bleeds, telangiectases, pulmonary and cerebral arteriovenous malformations, and gastrointestinal bleeding (MacAllister et al., 1994). The role of endoglin in hemopoietic cells is unknown. Its expression is restricted to a subset of erythroid progenitors in both adult and fetal marrow, and more recently, to subpopulations of pre-B cells in fetal marrow (Bühring et al., 1991; Rokhlin et al., 1995). Monocytes in culture and tissue macrophages also express endoglin, suggesting that it might play a specific role in the specialized function of these cells (Lastres et al., 1992; O'Connell et al., 1992).

Here, we have analyzed the role and regulated expression of endoglin in monocytic cells. To our surprise, TGF-β was found to upregulate the expression of endoglin not only on U-937 cells, but also on peripheral blood monocytes. Furthermore, the upregulation of endoglin on peripheral blood monocytes in culture can be abolished by a neutralizing antibody to TGF-β1, even in the absence of serum, suggesting that release of active ligand by monocytes might stimulate their expression of endoglin by an autocrine-like mechanism. We also observed that TGF-β1 not only increases the expression of endoglin, but also that of R-I, R-II, and betaglycan, suggesting that common mechanisms might govern the regulation of expression of the different TGF-β receptors. It remains to be determined if the increased mRNA expression is associated with a higher transcription rate or increased message stability. Higher levels of all four receptors were detected at the surface of U-937 cells which had been treated with ligand 72 h before cross-linking experiments with radiolabeled TGF-β1 (Lastres, P., unpublished data). A similar upregulation of TGF-β receptors might occur upon activation of monocytes in vivo at sites of inflammation or injury which would lead to further amplification of the response to TGF-β generally released at these sites.

We chose to transfect U-937 cells with endoglin to gain some understanding of its potential effects on the response of these monocytic cells to TGF-β (Table II). Endoglin, which binds the β1- but not the β2-isoform, specifically blocked the inhibitory action of TGF-β1 on proliferation of U-937 transfectants. Downregulation of c-*myc*, which is associated with an arrest in cell cycle, was not observed in the L-endoglin transfectants. Furthermore, the basal level of c-*myc* was increased by twofold, suggesting that endoglin was interfering with proliferation inhibition by preventing the downregulation of c-*myc*. This is in agreement with the finding that overexpression of c-*myc* blocks the growth inhibitory response to TGF-β1 (Alexandrow et al., 1995). S-Endoglin was less efficient in blocking the inhibitory effects of TGF-β1 on cell growth and an increase in the basal level of c-*myc*, similar to that seen in the L-endoglin transfectants, was observed in the S-endoglin transfectants; however, a certain decrease in the level of c-*myc* could be observed upon addition of ligand. The only difference between S-endoglin and L-endoglin is a shorter cytoplasmic domain and a lower level of serine/threonine phosphorylation of S-endoglin. Thus, the differences observed in the biological responses of both isoforms suggest an important role of the cytoplasmic region in endoglin function. In this sense, it is interesting to note that TGF-β1 is able to inhibit the constitutive phosphorylation of endoglin (Lastres et al., 1994). As the cytoplasmic domains of endoglin and betaglycan are highly conserved (Gougos and Letarte, 1990; Morén et al., 1992), these two proteins are likely to have similar mediators.

The presence of endoglin was also able to inhibit the increase in fibronectin synthesis and in the fibronectin-dependent cell adhesion seen in response to TGF-β. Furthermore, phosphorylation of PECAM-1 and the subsequent homotypic aggregation of these cells, were also inhibited by endoglin expression. By contrast, other biological responses to TGF-β such as the upregulation of TGF-β receptors (R-I, R-II, and betaglycan), changes in the expression of integrins ($\alpha_4\beta_1$ and $\alpha_5\beta_1$) and of adhesion proteins, members of the immunoglobulin superfamily (PECAM-1 and ICAM-1) were not altered in the endoglin transfectants. The distinct cellular responses observed suggest the existence of a mechanism of signal transduction finely regulated downstream of the R-I/R-II complex. The complexity of the TGF-β responses is such that one should not conclude that endoglin is always inhibitory. The interactions among TGF-β isoforms, modulators (betaglycan and endoglin), signalers (R-I and R-II), and probably additional components of the receptor and signaling systems, could account for the pleiotropic effects of TGF-β (Wahl, 1994; Moses et al., 1990).

Our studies clearly demonstrate that endoglin can modulate several of the responses to TGF-β1 and is thus an integral component of the receptor system. The exact molecular mechanism of action of endoglin remains to be determined. Coimmunoprecipitation in affinity labeled pig endothelial cells and human leukemic cells of endoglin, R-II, and R-I suggests that such a complex is formed (Yamashita et al., 1994; Zhang et al., 1996). We have also seen these heteromeric associations in human umbilical vein

Table II. Effects of Endoglin Overexpression on Cellular Responses Induced by TGF-β1

Biological response	U937/Mock	L-Endoglin	S-Endoglin
Growth inhibition	Yes	No	Minimal
c-myc			
Basal level		Increased	Increased
Downregulation	Yes	No	Partial
Stimulation			
Fibronectin synthesis	Yes	No	No
Cellular adhesion	Yes	No	No
PECAM-1 phosphorylation	Yes	No	No
Homotypic aggregation	Yes	No	No
Stimulation			
PECAM-1 expression	Yes	Yes	Yes
$\alpha_5\beta_1$ integrin expression	Yes	Yes	Yes
Reduction			
$\alpha_4\beta_1$ integrin expression	Yes	Yes	Yes
ICAM-1 expression	Yes	Yes	Yes
Stimulation of mRNA levels			
TGF-β1 (13–20-fold)	Yes	Yes	Yes
R-I, R-II, and betaglycan (5–20-fold)	Yes	Yes	Yes

Specific transcripts of c-*myc* were stimated by Northern blot analysis. Surface expression of PECAM-1, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and ICAM-1 were estimated by flow cytometry and levels of mRNA for TGF-β1 and TGF-β receptors were estimated by quantitative PCR.

endothelial cells. In endoglin transfectants of U-937 cells, the presence of a putative R-I coimmunoprecipitated with endoglin and the increased ligand binding to this receptor, point to an active role of endoglin in these heteromeric complexes. It can be postulated that this association with the signaling receptors might confer to endoglin the ability to bind TGF-β. This could explain that in endothelial cells, where endoglin expression is high (10^6 molecules per cell), in contrast with the low expression of signaling receptors (10^4 molecules per cell), only a small number of endoglin molecules bind TGF-β (Cheifetz et al., 1992). Unlike endoglin, betaglycan readily binds TGF-β even in its soluble form (López-Casillas et al., 1991), suggesting that endoglin has a mechanism of action different from betaglycan. This hypothesis is in agreement with the lack of growth inhibitory response of TGF-β in endoglin transfectants, whereas transfection of betaglycan confers increased inhibition of proliferation in the presence of ligand (López-Casillas et al., 1991). Also, these counteracting effects of endoglin and betaglycan might be compatible with the almost non-overlapping expression of these two proteins in several cell types.

Our results suggest that endoglin functions as an auxiliary receptor which contributes to the complex regulation of TGF-β responses.

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