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A Glycosylation-Deficient Endothelial Cell Mutant with Modified Responses to Transforming Growth Factor-β and Other Growth Inhibitory Cytokines: Evidence for Multiple Growth Inhibitory Signal Transduction Pathways

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An endothelial cell line (M40) resistant to growth inhibition by transforming growth factor-beta type 1 (TGFβ1) was isolated by chemical mutagenesis and growth in the presence of TGFβ1. Like normal endothelial cells, this mutant is characterized by high expression of type II TGFβ receptor and low expression of type I TGFβ receptor. However, the mutant cells display a type II TGFβ receptor of reduced molecular weight as a result of a general defect in N-glycosylation of proteins. The alteration does not impair TGFβ1 binding to cell surface receptors or the ability of TGFβ1 to induce fibronectin or plasminogen activator inhibitor-type I production. M40 cells were also resistant to growth inhibition by tumor necrosis factor alpha (TNFα) and interleukin-1 alpha (IL-1α) but were inhibited by interferon-γ (IFNγ) and heparin. These results imply that TGFβ1, TNFα, and IL-1α act through signal transducing pathways that are separate from pathways for IFNγ and heparin. Basic fibroblast growth factor was still mitogenic for M40, further suggesting that TGFβ1, TNFα, and IL-1α act by direct inhibition of cell growth rather than by interfering with growth stimulatory pathways.

INTRODUCTION

The regulation of new capillary blood vessel formation is thought to be under the control of growth-stimulatory and -inhibitory factors (Folkman and Klagsbrun, 1987). In vitro, these factors can modulate endothelial cell proliferation, migration, and synthesis of specific proteins. Although considerable progress has been made in understanding the mechanism of action of growth-stimulatory factors, most notably basic fibroblast growth factor (bFGF),[1] the mechanisms underlying growth inhibition remain more elusive.

The best known growth inhibitor, transforming growth factor-beta type 1 (TGFβ1), inhibits basal and bFGF-induced endothelial cell proliferation (Baird and Durkin, 1986; Fràter-Schroeder *et al.*, 1986) and cell migration (Heimark *et al.*, 1986; Müller *et al.*, 1987). TGFβ1 also stimulates extracellular matrix (ECM) formation in endothelial cells by inducing the synthesis of ECM proteins, such as fibronectin and types IV and V collagens (Müller *et al.*, 1987; Madri *et al.*, 1988). TGFβ1 further contributes to ECM formation by means of inhibiting the degradation of ECM proteins by specific proteases (increased production of plasminogen activator inhibitor-type 1 [PAI-1] and decreased plasminogen activator activity) (Saksela *et al.*, 1987; Pepper *et al.*, 1990). In most cells, TGFβ1 binds with high affinity to cell surface receptor glycoproteins of 53 kDa (type I) and 70–85 kDa (type II) and with lower affinity to 200–

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[1] Abbreviations used: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; ECM, extracellular matrix; EMS, ethyl methanesulfonate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IFNγ, interferon-gamma; IL-1α, interleukin-1 alpha; PAI-1, plasminogen activator inhibitor-type I; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TGFβ1, transforming growth factor-beta type 1; TNFα, tumor necrosis factor alpha; Tris, tris(hydroxymethyl)aminomethane.

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400 kDa (type III) receptors (Cheifetz *et al.*, 1988; Segarini *et al.*, 1989). Endothelial cells exhibit only the type I and type II receptors, with the latter being predominantly expressed (Fafeur *et al.*, 1990). Although the mechanisms by which TGFβ1 exerts its effects on endothelial cells are still poorly understood, available evidence suggests that the growth inhibitory effect is not caused by interference with known mitogenic signaling pathways (reviewed in Massagué, 1990; Sporn and Roberts, 1990). For example, TGFβ1 blocks the proliferation of endothelial cells without altering the signaling capacity of bFGF receptors (Fafeur *et al.*, 1991). Recent data suggest that growth arrest occurs in late G1 and that earliest effects of TGFβ1 involve regulation of genes controlling cell adhesion and enhanced formation of ECM (Massagué, 1990) and that TGFβ-induced growth inhibition may be linked to suppression of phosphorylation of the retinoblastoma gene product (Laiho *et al.*, 1990a). More recently, the type II TGFβ receptor of a porcine renal epithelial cell line was cloned and characterized as a serine/threonine kinase (Lin *et al.*, 1992), thus lending support to the notion that this receptor is involved in signaling through a novel pathway.

Other cytokines, including tumor necrosis factor-alpha (TNFα) (Fràter-Schroeder *et al.*, 1987), interleukin-1 alpha (IL-1α) (Cozzolino *et al.*, 1990), interleukin-6 (Böhlen *et al.*, 1987), and interferons (INFs) (Böhlen *et al.*, 1987; Friesel *et al.*, 1987) were also reported to inhibit endothelial cell proliferation. Although all these factors inhibit the growth of endothelial cells in a manner similar to that of TGFβ, their signal transducing mechanisms are most likely different, as evidenced by the considerable structural differences in the cytoplasmic domains of their respective receptors (Aguet *et al.*, 1988; Sims *et al.*, 1988; Smith *et al.*, 1990; Lin *et al.*, 1992).

We found it intriguing that several cytokines with apparently different mechanisms of action would all inhibit potently the growth of endothelial cells. In an attempt to understand better the mechanisms by which growth inhibition of endothelial cells is effected, we tried to produce a cell mutant that is resistant to the growth inhibitory activity of TGFβ1. Here we report the isolation of a glycosylation-deficient endothelial cell mutant that is resistant to TGFβ1-induced growth inhibition while still being responsive to some other actions normally mediated by this cytokine. This mutant also proved to be resistant to growth inhibitory activities of TNFα and IL-1α but not to that of IFNγ nor that of heparin, a well-documented but rather ill-characterized inhibitor of bovine endothelial cell growth.

MATERIALS AND METHODS

Chemicals

Porcine platelet TGFβ1 and human recombinant-IL-1α and -TNFα were purchased from R&D Systems (Minneapolis, MN). Bovine brain bFGF was isolated as described (Gospodarowicz *et al.*, 1984). A recombinant human bFGF analogue, $Glu^{3,5}$-$Ser^{78,96}$-bFGF, with biological activity being indistinguishable from native bFGF, was expressed in *Escherichia coli* and purified as described (Seddon *et al.*, 1991). Recombinant human IFNγ was a gift from Biogen (Geneva, Switzerland). Heparin was purchased from Hepar Industries (Franklin, OH). Ethyl methanesulfonate was purchased from Sigma (St. Louis, MO). Iodinated TGFβ1 was a gift of Dr. Anita Roberts (National Cancer Institute, Bethesda, MD) or purchased from New England Nuclear (Boston, MA) (both preparations had specific activities of 1.9–2.2 μCi/pmol). ^{35}S-protein labeling mix (77% ^{35}S-methionine, 18% ^{35}S-cysteine), D-[6-^{3}H(N)]-glucosamine hydrochloride, and D-(2-^{3}H)-mannose were purchased from New England Nuclear. Rabbit antiserum against human PAI-1 was kindly provided by Dr. Daniel Rifkin (New York University, NY), and rabbit antiserum against bovine fibronectin was purchased from Calbiochem (La Jolla, CA). Protein G-Agarose beads were from Genex (Gaithersburg, MD), and tunicamycin was purchased from Sigma.

Production of TGFβ1-Resistant Endothelial Cell Mutants

Endothelial cells from adult bovine aortic arch were prepared and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone, Logan, UT) as previously described (Gospodarowicz *et al.*, 1984). Preconfluent endothelial cells (passage 5) maintained in DMEM/10% calf serum (10 ml/100-mm plates) were treated for 24 h with 0.4 μl/ml ethyl methanesulfonate (EMS). The treatment resulted in the death of 80% of the cells. The remaining cells were washed and fed with fresh medium. Four days later, a total of 14×10^6 cells were split into 100-mm plates at 3×10^5 cells per plate in fresh medium. The following day, 2 ng/ml of TGFβ1 was added. The cells were fed fresh medium containing TGFβ1 every 5 d. After 2–3 wk of treatment, clonal cell lines were isolated by dilution cloning of the colony-derived cultures and expanded in the presence of 1 ng/ml bFGF and in the absence of TGFβ1. Cell lines were subjected to two additional rounds of selection for 7–10 d in TGFβ1, with 7- to 10-d periods of culture in TGFβ1-free/bFGF-containing medium between rounds of TGFβ1 selection. Resultant cell lines were periodically tested for resistance to TGFβ1-induced cell growth. Normal (or wild-type) endothelial cells that were not exposed to EMS and TGFβ1 selection but were grown in parallel with the mutant cells and were used at the same passage number (between passage 5 and 20) served as control cells. Additional control cells that were exposed or not exposed to EMS, and subjected to alternative treatments with TGFβ1 and bFGF as described above, were also cultured in parallel and used in most experiments. They gave comparable results to wild-type cells.

Cell Growth Assay

Cells were seeded in 24-multiwell plates at a density of 10 000 cells/0.5 ml of DMEM/10% calf serum per well. The following day, cells were fed growth factors or growth inhibitors. At different time intervals, cells were detached from the culture dishes with trypsin and counted in a Coulter particle counter (Coulter, Hialeah, FL).

Assays of Fibronectin and PAI-1 Production

Subconfluent cell cultures in 35-mm dishes were fed serum-free DMEM supplemented with or without 5 ng/ml of TGFβ1. After 15 h, the medium was replaced with cysteine- and methionine-free DMEM containing 30 μCi/ml ^{35}S-methionine/cysteine with or without 5 ng/ml of TGFβ1. After 5 h of labeling, the medium was removed and retained for further analysis. Cell monolayers were washed with ice-cold phosphate-buffered saline (PBS) and were extracted with 0.5 ml of a solution containing 1 mM urea, 1 mM dithiothreitol, 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 10 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mann-

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heim, Indianapolis, IN) according to a published procedure (Ignotz and Massagué, 1986). The supernatants of the cell extracts and samples of the media were lyophilized and diluted with 0.5 ml of 0.5% Triton X-100. A portion of the samples was retained for further analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Fibronectin and PAI-1 were immunoprecipitated from the diluted extracts with anti-fibronectin and anti-PAI-1 antibodies, respectively, as follows. Protein G-Agarose beads (10 μl) were incubated with an excess of anti-fibronectin or of anti-PAI-1 antibody solution (5 μl) at 4°C for 1 h. The beads were then washed once in 0.5% Triton X-100, and the medium or cell samples were added. Incubation was allowed to take place for 2 h at 4°C. The beads were then washed four times in 0.5% Triton X-100, 0.15 M NaCl, 25 mM Tris-HCl, pH 7.4. Fibronectin or PAI-1 were released by heating at 100°C for 2 min in electrophoresis sample buffer and electrophoresed on a 6%-SDS polyacrylamide gel. Gels were fixed and treated with an autoradiographic enhancer (Amplify; Amersham, Arlington Heights, IL) according to the manufacturer's suggestion, dried, and exposed for autoradiography.

Receptor Binding Assay

Binding of ^{125}I-TGFβ1 to cell monolayers was performed as described previously (Fafeur *et al.*, 1990). Subconfluent monolayers of endothelial cells in 24-multiwell plates were incubated in binding buffer (DMEM, pH 7.4, containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] and 0.1% bovine serum albumin) for 2 h at 37°C. Various concentrations of ^{125}I-TGFβ1, with or without 5 nM unlabeled TGFβ1, were added and incubation allowed for another 2 h at room temperature. Cultures were then washed four times with binding buffer and solubilized with a solution containing 1% Triton X-100, 10% glycerol, HEPES 25 mM, pH 7.4. Nonspecific binding measured in the presence of 5 nM unlabeled TGFβ1 was subtracted from total binding to determine specific binding. The data were analyzed by the method of Scatchard (1949).

Affinity-Labeling Assay

Affinity-labeling of subconfluent endothelial cell monolayers with ^{125}I-TGFβ1 and disuccinimidyl suberate (DSS) was performed as previously described (Fafeur *et al.*, 1990). Briefly, cultures were incubated with 100 pM ^{125}I-TGFβ1, in the presence or absence of 5 nM unlabeled TGFβ1, for 3–5 h at 4°C. Cultures were washed and cross-linked using 0.5 mM DSS. The cells were then detached from the plates with a cell scraper in ice-cold sucrose buffer containing 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin (Boehringer Mannheim), and 1 mM PMSF. After centrifugation at 13 000 rpm, the pellets were resuspended in 50 mM Tris-HCl, pH 6.9, 2% SDS, 1% glycerol, and 10% 2-mercaptoethanol. The samples were boiled for 2 min and electrophoresed on 6% SDS polyacrylamide gels. Gels were stained for protein with Coomassie Blue, dried, and exposed to film with enhancing screens.

Metabolic Labeling With Radioactive Sugars

Subconfluent cell cultures in 35-mm wells were fed serum-free DMEM containing 50 μCi/ml of ^{3}H-mannose or 50 μCi/ml of ^{3}H-glucosamine. After 18 h at 37°C, cells were washed twice with PBS, extracted in sucrose buffer, and analyzed by 6% SDS polyacrylamide gel electrophoresis (see above, Affinity-Labeling Assays).

RESULTS

Isolation of an Endothelial Cell Mutant Resistant to TGFβ1-Induced Growth Inhibition

Previous studies have described the isolation of TGFβ-resistant clones from a mink lung epithelial cell line (Chinkers, 1987). We used the same strategy, which consisted in maintaining sparse cell cultures in the presence of 2 ng/ml TGFβ1 after chemical mutagenesis of the cells and isolating cell colonies that grew under these conditions. Although cells treated with TGFβ1 for 2–3 wk were usually able to resume proliferation after replating in fresh TGFβ1-free media, a significant portion of colonies failed to grow after cloning. We found that the rate of survival could be improved when isolated TGFβ1-resistant colonies were subsequently grown in the presence of bFGF (1 ng/ml). After treatment with EMS, TGFβ1-resistant clones of endothelial cells were obtained and taken through two additional cycles of TGFβ1-resistance selection interrupted by bFGF-growth stimulation periods. Of 35 TGFβ1-resistant colonies originally isolated from 14×10^6 mutagenized endothelial cells, only one clone (M40) remained completely resistant to TGFβ1 for over 6 mo in culture and was further characterized. Another TGFβ1-resistant clone had characteristics similar to those of the M40 clone (a modified TGFβ type II receptor, see below) but had very poor growth properties and did not survive after several months in culture.

Exposure of sparsely cultured nonmutagenized control endothelial cells (4.2×10^6 cells tested) to 2 ng/ml TGFβ1 for 2–3 wk resulted in complete growth arrest of all cells, and no cells resistant to TGFβ1-induced growth inhibition emerged.

Defect in Growth-Inhibitory Response

The growth-inhibitory response of normal endothelial cells to TGFβ1 was half-maximal at 0.05 ng/ml and maximal at 1 ng/ml (Figure 1). The mutant M40 cells were unresponsive to TGFβ1 concentrations of up to 10 ng/ml (Figure 1), and the rate of proliferation was

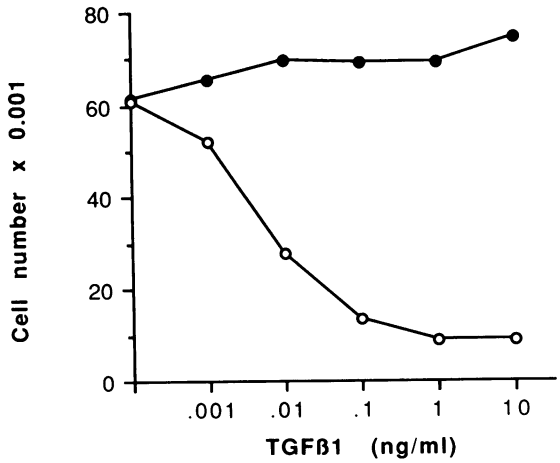

Figure 1. Inhibition of cell proliferation by TGFβ1 in normal (○) and M40 mutant (●) endothelial cells. Duplicate samples were counted on day 6 after plating. Individual values deviated <10% from averages.

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essentially the same in the presence or absence of 1 ng/ml of TGFβ1 (Figure 2). M40 cells, like normal endothelial cells, were responsive to the growth-promoting activity of bFGF (4 ng/ml) (Figure 2). TGFβ1, which is a potent inhibitor of bFGF-induced endothelial cell proliferation (Baird and Durkin, 1986; Fràter-Schroeder *et al.*, 1986), had no effect on bFGF-stimulated M40 cells (Figure 2).

Resistance to Other Endothelial Growth Inhibitors

In control experiments, we established that near maximal growth-inhibitory responses were obtained when endothelial cells were incubated with the inhibitors TNFα (1 ng/ml), IL-1α (10 ng/ml), IFNγ (10 ng/ml), and heparin (10 μg/ml) (Figure 3). At those concentrations, M40 cells were not inhibited by TNFα and IL-1α (Figure 3, left), but normal growth inhibition occurred with INFγ and heparin (Figure 3, right). This selective resistance to the various growth inhibitors was also found when M40 cells were growth stimulated by bFGF.

Morphological Changes in Response to TGFβ1

Normal endothelial cells show dramatic morphological changes in response to TGFβ1, involving an enlarged and flattened shape (Chinkers, 1987) (Figure 4). In contrast, M40 cells did not change shape in response to TGFβ1 (Figure 4). In the absence of TGFβ1, both cell types had a cobblestone appearance at confluence, characteristic of endothelial cells.

TGFβ1-Induced Synthesis of Extracellular Matrix Proteins

To determine whether the observed lack of growth inhibition by TGFβ1 is the result of a general impairment of the signal transducing mechanism, we tested whether other cellular responses to TGFβ1 were altered in the mutant endothelial cells. In this context, TGFβ1 can affect cell adhesion by enhancing synthesis and deposition of extracellular matrix components, such as fibronectin (Ignotz and Massagué, 1986; Müller *et al.*, 1987). We found that when biosynthetically labeled normal and mutant endothelial cells were treated with TGFβ1, increased secretion of a 220-kDa protein, identified as fibronectin by immunoprecipitation, was observed with both types of cells (Figure 5). Furthermore, in a similar experiment, the secretion of PAI-1, another TGFβ1-inducible protein thought to be involved in the regulation of extracellular matrix production (Sprengers and Kluft, 1987), was clearly stimulated in TGFβ1-treated normal and mutant cells (Figure 5). Comparable results were obtained when fibronectin and PAI-1 were determined in cell extracts rather than in conditioned media, suggesting that TGFβ1 stimulated the synthesis of these proteins both in normal and mutant cells. These data indicate that at least some TGFβ1-associated signaling

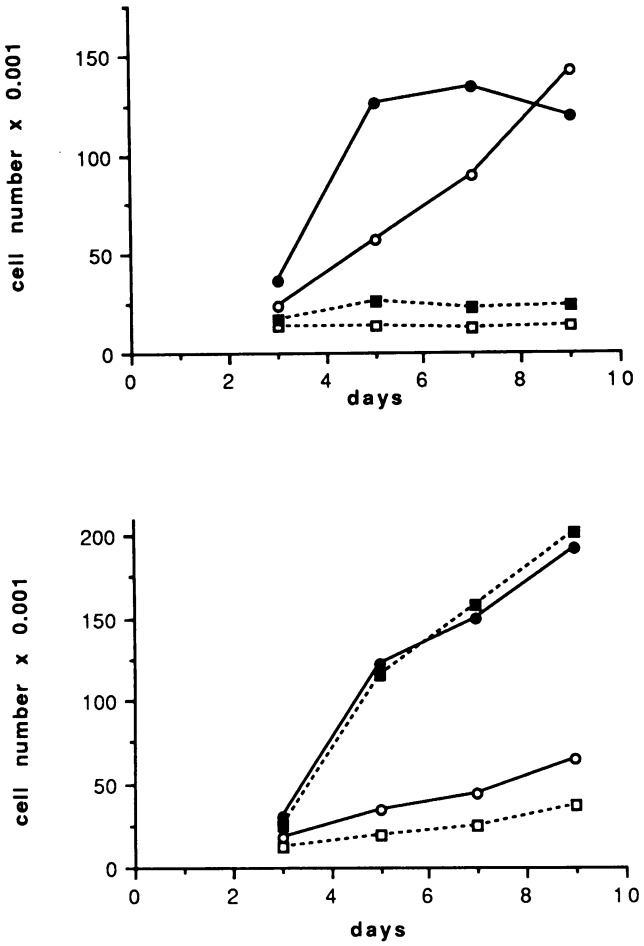

Figure 2. Growth curves of normal (top) and M40 (bottom) endothelial cells without added factors (○), in the presence of 1 ng/ml TGFβ1 (□), in the presence of 4 ng/ml bFGF (●), and with a combination of 1 ng/ml TGFβ1 and 4 ng/ml bFGF (■). Points represent duplicate samples. Individual values deviated <10% from averages. Although the results shown indicate that TGFβ1 alone slightly inhibited growth of M40 cells, this was not confirmed in two other experiments (e.g., see Figure 3; TGFβ1 has a slight stimulatory effect).

pathways remained intact in the M40 cell mutant. However, levels of fibronectin and PAI-1 were reproducibly lower in mutant cells, as compared with normal endothelial cells.

Characterization of TGFβ Receptors

In previous experiments (Fafeur *et al.*, 1990), two types of TGFβ receptors of ~65 and 85 kDa (type I and II receptors, respectively) were identified by affinity labeling techniques in bovine endothelial cells. Similar experiments performed in this study with normal and M40 cells showed that both cell types contained the same receptor types, although the type II receptor in normal endothelial cells appeared to be closer to 90 kDa (Figure 6). As previously observed, that 85–90 kDa type

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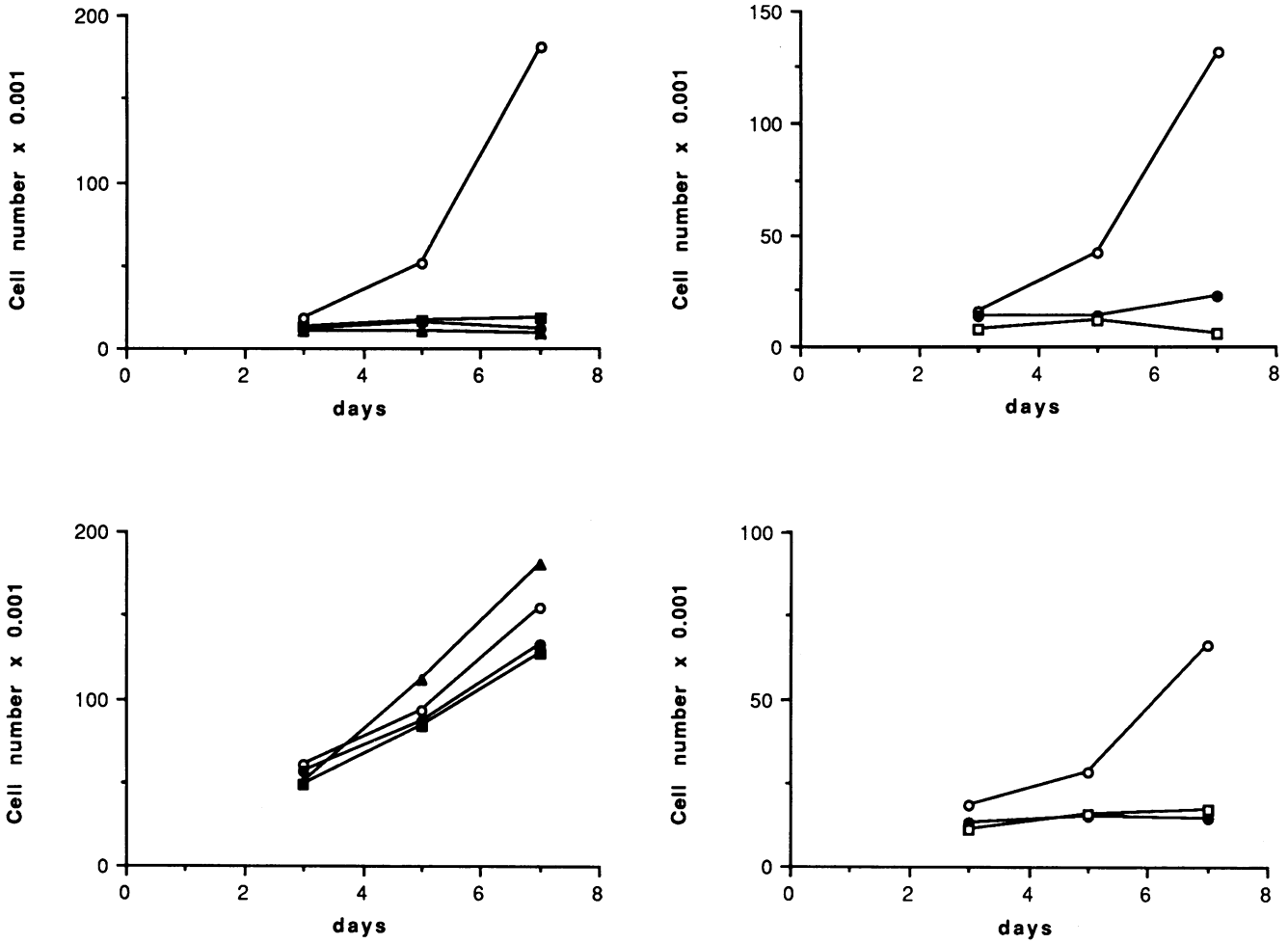

Figure 3. Growth curves of normal (top) and mutant M40 (bottom) endothelial cells. Left, growth in the absence of added factor (○), TGFβ1 (1 ng/ml) (▲), TNFα (1 ng/ml) (■), and IL-1α (10 ng/ml) (●) are shown. Right, growth curves in the absence of added factors (○), IFNγ (10 ng/ml) (□), and heparin (10 μg/ml) (●). Cells were counted in duplicate after various times as indicated. Individual values deviated <10% from averages.

II receptor is the predominant form in normal cells, with the 65-kDa type I receptor sometimes being barely detectable (Fafeur *et al.*, 1990) (Figure 6). M40 shows the same receptor distribution; however, the type II receptor is clearly of lower molecular weight than that of normal cells (85 vs. 90 kDa). In addition, this type II receptor migrates as a much broader species, suggesting that there is heterogeneity in the glycosylation pattern of this receptor. The type I receptor also appears to be slightly smaller than the corresponding normal receptor (Figures 6 and 7).

Modification of TGFβ Receptor Glycosylation

Exposure of cells to increasing concentrations of tunicamycin, a metabolic inhibitor of N-linked glycosylation (Fuhrmann *et al.*, 1985), resulted in a dose-dependent loss of the type II receptor in normal (90-kDa band) and M40 (85-kDa band) cells. Instead, a band corresponding to a smaller cross-linked protein of ~75 kDa appeared in tunicamycin-treated normal and mutant endothelial cells (Figure 7). These data indicate that a structural alteration of the type II receptor in mutant cells is located within the glycosylated part of this receptor. We were not able to conclude from our gel data whether tunicamycin treatment also caused a glycosylation defect in the type I receptor. Differences in migration of this receptor from tunicamycin-treated and control cells are expected to be small (5 kDa) (Cheifetz *et al.*, 1988) and, in our hands, were too small to be assessed reliably.

Given this evidence for a structural alteration of the predominant type II receptor in mutant endothelial cells, we investigated the binding characteristics of TGFβ1 by Scatchard analysis. TGFβ1 bound about equally to endothelial cell surface receptors of both normal and M40

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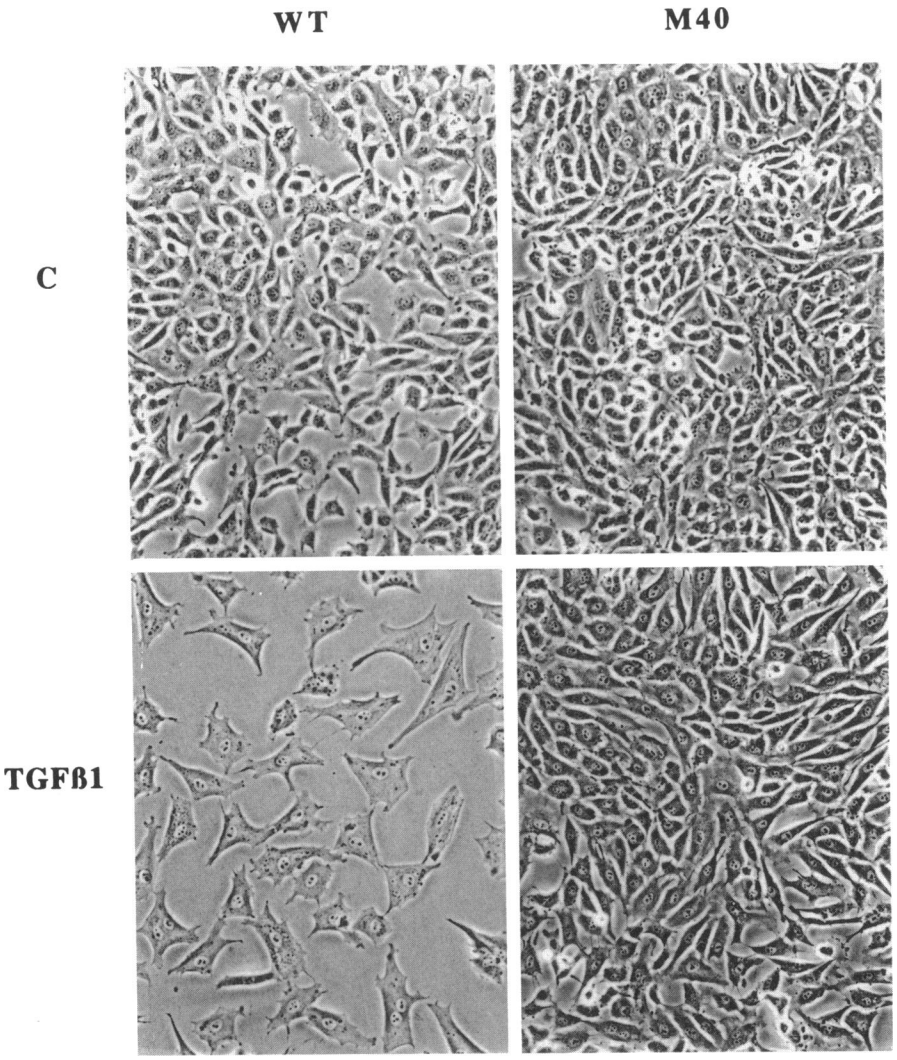

Figure 4. Effect of TGFβ1 on the morphology of normal and mutant M40 endothelial cells. Cells were seeded at a low density (50 000 cells per 35-mm dish) and fed the following day with TGFβ1 (1 ng/ml) or control buffer (C). Phase-contrast photomicrographs of the cultures were taken after 5 d of growth. Magnification ×160.

cells. Scatchard analysis of the data revealed that M40 cells expressed one high-affinity TGFβ-binding site (25 200 sites/cell, K_d = 7.0 pM). These results are comparable with those obtained with normal endothelial cells (19 300 sites/cell, K_d = 6.3 pM) (Figure 8).

Evidence for a General Glycosylation Defect

We examined the ability of normal and M40 cells to incorporate radioactive sugars into the carbohydrate chains of proteins. When cells were labeled with ^{3}H-glucosamine, cellular proteins in M40 cells had a significantly higher mobility in SDS gel electrophoresis than proteins from normal cells (Figure 9). When cells were labeled with ^{3}H-mannose, labeled proteins were detectable at much lower levels in M40 cells than in normal cells (Figure 9). These results indicate that the M40 cells have a general glycosylation defect. The results, obtained when cells were incubated for 24 h with tunicamycin (Figure 7), further suggest that this glycosylation defect affects at least the processing of N-linked carbohydrate chains of proteins.

DISCUSSION

The data presented here are discussed in the context of two main issues. First, it is intriguing that responses to TGFβ1 appear to be differentially impaired in M40 mutant endothelial cells. We observed that these cells cannot respond any longer to the normally growth inhibitory signal of TGFβ1 but remain able to signal the induction of the synthesis of specific proteins, fibronectin, and PAI-1, both well known for their TGFβ1-responsiveness.

Increasing evidence suggests that the type I and/or type II receptors, but not the type III receptor, are responsible for transducing the growth regulatory effect of TGFβ1. Some cell types, such as bovine endothelial cells, express only the type I and type II receptors and are responsive to various actions of TGFβ1, such as

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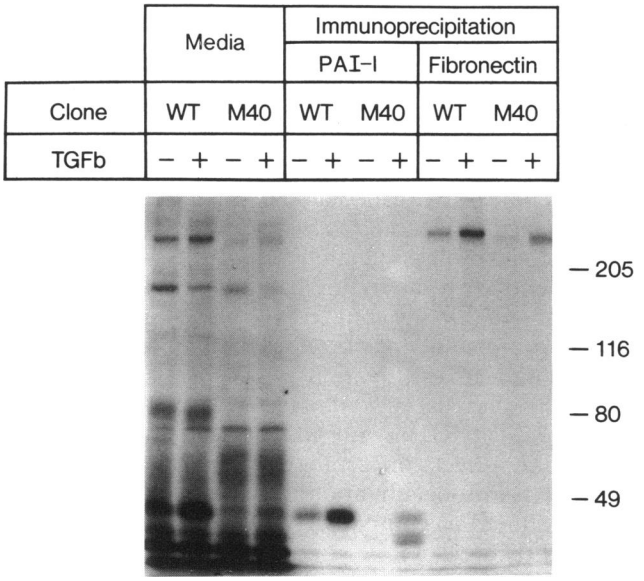

Figure 5. Effect of TGFβ1 on the amounts of fibronectin and PAI-1 in the conditioned media of normal (WT) and mutant (M40) endothelial cells. Cells were incubated with (+) or without (−) 5 ng/ml of TGFβ1 for 15 h and labeled for the last 5 h with ^{35}S-methionine/cysteine mixture. Conditioned media were directly analyzed by 6% SDS-polyacrylamide gel electrophoresis, either before or after immunoprecipitation with antibodies against PAI-1 or fibronectin. Equal numbers of counts from control and TGFβ-treated cultures (~9 × 10^6 cpm) were loaded into each lane. Note that the molecular weight of fibronectin from mutant cells is slightly lower than that of fibronectin synthesized by normal endothelial cells. An additional PAI-1-like immunoreactive protein of ~40 kDa was consistently detected in M40 cells but not in normal endothelial cells.

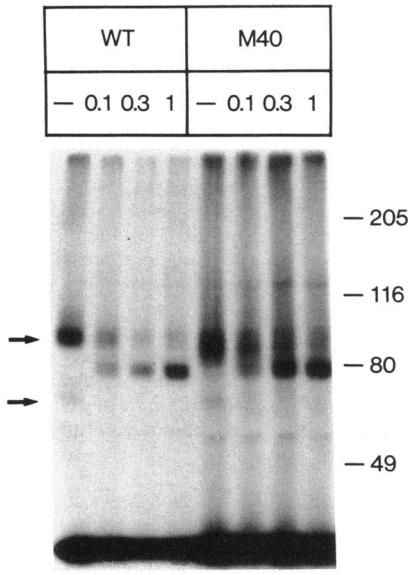

Figure 7. Effect of tunicamycin on TGFβ receptor subtypes in normal (WT) and mutant (M40) endothelial cells. Cells were incubated for 24 h with various concentrations of tunicamycin (0.1, 0.3, 1 μg/ml). Samples were then affinity-labeled with ^{125}I-TGFβ1 and processed as indicated in Figure 6.

growth inhibition or induction of proteins involved in controlling extracellular matrix modeling. Previous experiments with mink lung epithelial cell mutants that lack either type I or type I and type II receptors showed that those cells were refractory to growth inhibition by TGFβ1 and other TGFβ-mediated cellular responses, such as elevated production of fibronectin (Boyd and Massagué, 1989; Laiho *et al.*, 1990b,c). It was concluded that the type I and type II receptors are necessary for those responses to TGFβ1 and it was suggested that both receptors may be coordinately expressed as part of a common signaling TGFβ1 receptor complex (Laiho *et al.*, 1990b,c). However, mutants lacking only the type

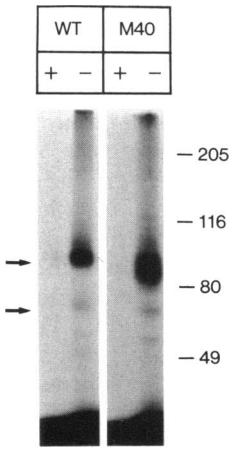

Figure 6. Autoradiograms of ^{125}I-TGFβ1 cross-linked to normal (WT) and mutant (M40) endothelial cell surface proteins. Subconfluent cells were affinity-labeled by incubation with 100 pM ^{125}I-TGFβ1 alone (−) or in combination (+) with 5 nM unlabeled TGFβ1, followed by treatment with disuccinimidyl suberate. Membrane extracts were analyzed by electrophoresis on 6% SDS-polyacrylamide gels. The positions corresponding to molecular size markers are indicated. Arrows indicate bands corresponding to the 90- and 65-kDa labeled cross-linked proteins (the molecular size refers to the receptor-ligand complexes).

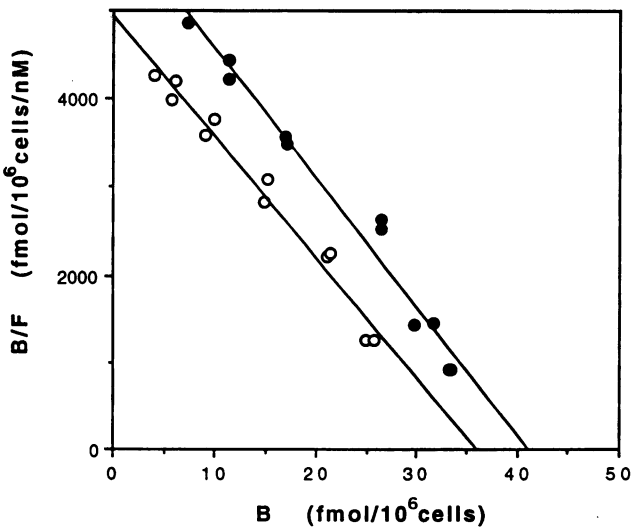

Figure 8. Scatchard plots of the binding of TGFβ1 to normal (○) and M40 (●) endothelial cells. Subconfluent cells (50 000 cells per well) were incubated with various concentrations of ^{125}I-TGFβ1 for 2 h at room temperature. Nonspecific binding was determined in the presence of 5 nM unlabeled TGFβ1.

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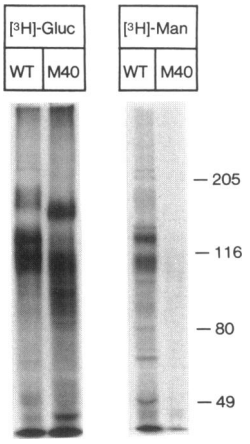

Figure 9. Incorporation of radioactive sugars into proteins in normal (WT) and mutant (M40) endothelial cells. Cells were labeled for 18 h with either ^{3}H-glucosamine (^{3}H-Gluc) or with ^{3}H-mannose (^{3}H-Man). Samples were analyzed by 6% SDS-polyacrylamide gel electrophoresis. The volumes of cell extracts loaded were adjusted so that the two lanes would contain a similar amount of tritium counts (2×10^5 and 2.5×10^4 cpm per lane for ^{3}H-glucosamine and ^{3}H-mannose, respectively). Coomassie blue staining of gels indicated that normal and mutant cell lines contained similar amounts of protein.

II receptor have not been found in these studies, and it is therefore difficult to assess the precise role of the type II receptor in TGFβ1 signal transduction. Other evidence implicates more specifically the type II receptor in transmitting TGFβ1 growth inhibitory effects. By investigating the mechanism by which bFGF counteracts the ability of TGFβ1 to inhibit DNA synthesis in bovine endothelial cells, we found that expression of the type II receptor (which is the major TGFβ1 receptor on endothelial cells) is selectively down-regulated by bFGF (Fafeur *et al.*, 1990). Furthermore, a recent study showed that in a hybrid cell line obtained by fusion of two human carcinoma cell lines that are not growth-inhibited by TGFβ1, growth inhibitory response to this cytokine was restored (Geiser *et al.*, 1992). In contrast, TGFβ1-mediated activation of fibronectin, collagenase, and PAI-1 gene expression occurred in both hybrid and parental tumor cells. The hybrid cells expressed large quantities of the type II receptor as compared with the parental carcinoma cells. From these experiments, it was concluded that the type II receptor is necessary for mediating the effects of TGFβ1 on inhibition of growth but not on gene activation of the hybrid cells (Geiser *et al.*, 1992).

In M40 mutant cells, the type II receptor is clearly structurally altered by aberrant glycosylation. At present, we cannot rule out the possibility that the type I receptor has a similar glycosylation defect (see RESULTS). Regardless, the fact that by Scatchard analysis we found no reduction of TGFβ1 binding to the mutant cells suggests that TGFβ1 binds to glycosylation-defective TGFβ receptors, as well as to their normal counterparts. Assuming that normal ligand-receptor binding is required for initiation of a normal signaling cascade, it is therefore likely that growth-inhibitory signaling is impaired downstream from receptor-ligand binding.

The glycosylation defect in M40 cells may interfere with other signaling mechanisms that are not directly related to the functionality of the TGFβ receptor(s). The finding that synthesis of fibronectin and PAI-1 is still induced in M40 cells shows that at least part of the TGFβ1-mediated signal transduction system is intact in M40 mutants. The differential effects could be the result of either separate transduction mechanisms mediated by type I and type II receptors, triggering either growth inhibition or specific protein synthesis, or of a common initial signal pathway mediated by these receptors, split downstream and leading to multiple actions. Whatever the mechanism, it is of interest that the results of the experiments with the hybrid carcinoma cell lines mentioned above (Geiser *et al.*, 1992) also suggest the existence of independent processes leading to growth inhibition and gene activation by TGFβ1. Furthermore, in Mv1Lu epithelial cells transfected to express the large T antigen, TGFβ1 is no longer growth inhibitory, whereas its effects on protooncogene c-jun induction and PAI-1, fibronectin, and thrombospondin synthesis are not altered as compared with parental cells (Laiho *et al.*, 1991).

The second issue concerns the differential response of mutant endothelial cells to various growth inhibitors, of which modes of action are largely unknown. The cytokines TGFβ1, TNFα, IL-1α, and IFNγ all potently inhibit growth of normal endothelial cells. In contrast, with the exception of IFNγ, these cytokines are without growth-inhibitory effects on M40 cells. The TNF, IL-1, and IFNγ receptors, like the TGFβ receptors, are glycoproteins, and their structures are unrelated (Aguet *et al.*, 1988; Sims *et al.*, 1988; Smith *et al.*, 1990; Lin *et al.*, 1992). Furthermore, their cytoplasmic domains do not contain sequences typical of protein tyrosine kinases, and this suggests that these cytokines may act on cell growth through novel signal transducing pathways. A possible interpretation of our data is that separate transducing pathways are triggered by these cytokines and are all impaired in the mutant cells. Alternatively, the similar loss of growth inhibitory responses to TGFβ1, TNFα, and IL-1α by M40 cells, both in the presence or absence of bFGF, suggests that the common growth inhibitory actions of these cytokines, triggered by unrelated ligand-specific receptors, may nevertheless be mediated by shared elements of their signaling pathways. In both cases, the general N-glycosylation defect in M40 cells suggests that protein(s) that must be N-glycosylated to function normally may be involved.

The fact that M40 cells remain growth inhibited by IFNγ suggests a separate inhibitory mechanism. Although very little is known about the signaling mechanisms of the various inhibitory factors, some other data support the concept of multiple inhibitory pathways. For example, increasing evidence suggests that IFNγ may interfere directly with growth stimulatory mechanisms induced by growth factors, including FGF (Friesel *et al.*, 1987; Hosang, 1988; Mundschau and Faller, 1991), in contrast to TGFβ1, which does not appear to inhibit growth factor-induced events (see Sporn and Roberts, 1990).

Bicara Ex. 1052
Bicara v. JHU - PGR2026-00025

Our data regarding the action of heparin on bovine endothelial cells also support the concept of multiple inhibitory mechanisms. Although the growth inhibitory activity of heparin on some cell types, e.g., smooth muscle cells and bovine endothelial cells, is well documented, its mechanism of action is unknown and generally not thought to be related to those of the cytokines. However, heparin action also may be mediated, at least in part, by typical signaling transduction pathways (Pukac *et al.*, 1992).

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Bicara Ex. 1052
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