Altered Structure of the Hybrid Cell Surface Proteoglycan of Mammary Epithelial Cells in Response to Transforming Growth Factor-β

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Abstract. Transforming growth factor beta (TGF-β) is a polypeptide growth factor that affects the accumulation of extracellular matrix by many cell types. We have examined the ability of mouse mammary epithelial (NMuMG) cells to respond to TGF-β and assessed the effect of the growth factor on the expression of their cell surface heparan sulfate/chondroitin sulfate hybrid proteoglycan. NMuMG cells respond maximally to 3 ng/ml TGF-β and the response is consistent with occupancy of the type III receptor. However, cells that are polarized, as shown by sequestration of the cell surface PG at their basolateral surfaces, must have the growth factor supplied to that site for maximal response. Immunological quantification of proteoglycan core protein on treated cells suggests that the cells have an unchanging number of this proteoglycan at their cell surface. Nonetheless, metabolic labeling with radiosulfate shows a $\sim$2.5-fold increase in $^{35}SO_4$-glycosaminoglycans in this proteoglycan fraction, defined either by its lipophilic, antigenic, or cell surface properties. Kinetic studies indicate that the enhanced radiolabeling is due to augmented synthesis, rather than slower degradation. Analysis of the glycosaminoglycan composition of the proteoglycan shows an increased amount of chondroitin sulfate, suggesting that the increased labeling per cell may be attributed to an augmented synthesis of chondroitin sulfate glycosaminoglycan on the core protein that also bears heparan sulfate, thus altering the proportions of these two glycosaminoglycans on this hybrid proteoglycan. We conclude that TGF-β may affect NMuMG cell behavior by altering the structure and thus the activity of this proteoglycan.

TRANSFORMING growth factor beta (TGF-β)[1] is a 25-kD polypeptide dimer found in serum and produced in vitro and in vivo by cells from connective tissue, epithelia, and the immune system (reviewed by Massagué, 1987; Sporn et al., 1987). The monomer can be of two types, termed β_1 or β_2, and homodimers of these often, but not always, have similar effects. TGF-β binds to at least three cell surface receptors (Cheifetz et al., 1987; Segarini et al., 1987) and elicits changes in the behavior of different cell types, most notably altered cell proliferation and differentiation. Its effects appear similar in vivo, where, for example, it prevents the outgrowth of proliferating mammary end buds (Silberstein and Daniels, 1987). The mechanisms whereby the factor's effects are produced are not known, but have attracted considerable attention as being central to an understanding of normal organ development, wound healing, and tumorigenesis.

Although seemingly unrelated to soluble growth factors, cell–extracellular matrix (ECM) interactions also influence cell behavior, affecting cell adhesion and motility, growth,

and differentiation (Wicha et al., 1982; Haeuptle et al., 1983; Blum et al., 1987). Interactions with the ECM involve a variety of matrix receptors, several of which, e.g., laminin receptor, integrins, cell surface proteoglycan (PG), have been implicated in binding the cytoskeleton (Brown et al., 1983; Woods et al., 1985; Tamkun et al., 1986; Rapraeger et al., 1986). These receptors are believed to influence cell behavior via translation of matrix support into cytoskeletal organization and cell shape. The degree to which these receptors translate ligand occupancy directly into other cytoplasmic responses remains unknown.

Treatment of cultured cells with TGF-β affects matrix accumulation. Many cells of mesenchymal and epithelial origin whose proliferation and differentiation are affected by TGF-β show elevated expression of fibronectin (FN), collagens and other cell-adhesion proteins (Ignotz and Massagué, 1986), apparently via regulation of mRNA levels (Ignotz et al., 1987). Chondroitin sulfate PG synthesis is also reported to be affected. Human smooth muscle cells (Chen et al., 1987) respond to TGF-β with increased amounts of chondroitin sulfate PGs in the cell layer and culture medium. Similarly, a variety of cell lines, including mink lung epithelial cells, show increased chondroitin sulfate PG in the ECM and culture medium in response to TGF-β (Bassols and Massagué, 1988).

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1. *Abbreviations used in this paper:* CM-PBS, calcium- and magnesium-containing phosphate-buffered saline; CMF-PBS, calcium- and magnesium-free PBS; ECM, extracellular matrix; FN, fibronectin; GAG, glycosaminoglycan; PG, proteoglycan; TGF-β, transforming growth factor (type β); TUT, Tris/Urea/Triton.

TGF-β may affect matrix receptors. Preadipocytes treated with TGF-β show an enhanced expression of integrin, an FN receptor, via regulation at pretranslational and posttranslational processing steps (Ignotz and Massagué, 1987). Thus, the growth factor has the potential to affect cell–matrix interactions not only by altering matrix accumulation, but also by changing the expression of receptors for the matrix.

Cell surface PGs are involved in matrix recognition. Cell surface PGs bearing heparan sulfate bind a variety of matrix components, localize to matrix-rich sites of cell adhesion and colocalize with the intracellular cytoskeleton (Höök et al., 1984; Lark and Culp, 1984; Woods et al., 1984, 1985; Rapraeger et al., 1986). A direct role for heparan sulfate PG in growth control has also been proposed (Ishihara et al., 1987). We have studied a cell surface PG of mouse mammary epithelial cells. This PG (a) is an integral membrane protein (Rapraeger and Bernfield, 1983), (b) is localized as a basolateral antigen to the site of matrix accumulation of polarized epithelial cells (Rapraeger et al., 1986), (c) contains primarily heparan sulfate in addition to a minor amount of chondroitin sulfate glycosaminoglycan (GAG) (David and Van den Berghe, 1985; Rapraeger et al., 1985), (d) binds matrix components, including FN (Saunders and Bernfield, 1988), interstitial collagens (Koda et al., 1985) and thrombospondin (Sun, X., D. Mosher, and A. Rapraeger, unpublished results), primarily via its heparan sulfate chains, and (e) interacts with the cytoskeleton (Rapraeger et al., 1986). This report examines the PG after culture in TGF-β to better understand the controls imposed on its cell surface expression.

Materials and Methods

Cell Culture and Radiolabeling

NMuMG mouse mammary epithelial cells (passages 13–25) were maintained in bicarbonate-buffered DME (Gibco Laboratories, Grand Island, NY) containing 10% FBS (Tissue Culture Biological, Tulare, CA) as described previously (David and Bernfield, 1979). Cells were plated at 10–25% of confluent density on tissue culture plastic dishes (Falcon Labware, Oxnard, CA) and used the next day for assessment of sparse cultures or cultured for 3–4 d for assessment of newly confluent cultures. Cells were also plated at 100% of confluence on plastic dishes or, for Fig. 3, filter chambers (Transwells, 24 mm, 0.4 µm, No. 3412; Costar, Cambridge, MA), and cultured for a week for assessment of postconfluent cultures. Cultures were radiolabeled for up to 48 h in low sulfate medium (David and Bernfield, 1979) containing 20–100 µCi/ml ^{35}SO$_4$ (ICN Radiochemicals, Irvine, CA). Cell morphology was recorded on Kodak plus-X 35-mm film using a Leitz inverted phase microscope.

TGF-β Isolation and Purification

Human TGF-β$_1$ was either kindly donated by Dr. Lynn Allen-Hoffman (Department of Medicine, University of Wisconsin, Madison, WI) after isolation from outdated human platelets and purification as described by Assoian et al., (1983) or obtained from R & D Systems, Inc. (Minneapolis, MN). Lyophilized aliquots were suspended in 4 mM HCl containing 1 mg/ml BSA and diluted immediately in culture medium or flash-frozen in liquid nitrogen and stored at −70°C.

PG Isolation and Purification

Isolation from Monolayers of NMuMG Cells. After a 24-h incubation in radiosulfate with or without TGF-β, the culture medium was removed and the cells recovered by scraping into calcium- and magnesium-free PBS (CMF–PBS) containing 0.5 mM EDTA and centrifuging (200 g). The supernatant containing extracellular PG was saved and the cells were washed by resuspension in CMF–PBS. The cells were either (a) extracted directly in 4 M guanidinium chloride containing 0.1% Triton X-100 and 10 mM Tris-HCl (pH 8.0) for quantification of lipophilic PG (see Fig. 4), (b) treated with bovine trypsin (Sigma Chemical Co., St. Louis, MO) for removal of cell surface PG (Rapraeger and Bernfield, 1985) followed by extraction of the cell residue in 0.1% Triton X-100, 8 M urea and 10 mM Tris-HCl (TUT, pH 8.0), or (c) initially extracted with isotonic, 1% Triton X-100 at pH 5.0 (Rapraeger et al., 1986). This latter treatment releases soluble intracellular components but leaves the intact cell surface PG anchored in the residue of the plasma membrane. Subsequent treatment with the low pH, isotonic Triton solution containing 0.5 M KCl releases the cell surface PG from the sedimentable residue (Rapraeger et al., 1986).

Purification. PG was purified by chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ). PG dialyzed into 50 mM sodium acetate (pH 4.5), 0.2 M NaCl, 8 M urea, and 1% Triton X-100 was applied to a 5-ml packed bed of resin and eluted in the same buffer with a 100 ml gradient of NaCl ranging from 0.2–1.0 M. The eluted PG was dialyzed into Tris-buffered (50 mM, pH 8.0) isotonic saline, applied to an antibody affinity column generated by coupling mAb 281, a monoclonal antibody specific for the PG core protein, to cyanogen bromide–activated Sepharose 4B (Pharmacia Fine Chemicals) and eluted in saline containing 50 mM ethanolamine (pH 11.5) as described by Jalkanen et al. (1985). The pH of the eluted PG was immediately adjusted to neutrality.

PG Quantification

Total ^{35}S-labeled GAG. Radiosulfate-labeled materials in CMF–PBS or TUT were spotted on cationic nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Richmond, CA) sandwiched in a dot blot apparatus (Microfold, V & P Scientific, San Diego, CA). The blot was washed briefly in 50 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl followed by 95% ethanol, dried and individual dots were assessed by scintillation counting (Beckman LS-5800 detector) in BioSafe (Research Products International, Mount Prospect, IL) cocktail. Spotting of various dilutions demonstrated that retention of PG or GAG by the membrane was >95% for these experiments, whereas inorganic sulfate and sulfated lipids failed to bind (Rapraeger, A., unpublished results).

To quantify chondroitin sulfate in fractions from DEAE–Sephacel chromatography, the column was run in the absence of urea and fractions were brought to final concentrations of 100 mM Tris-HCl (pH 8.0), 0.15 M NaCl. The samples were then treated at 37°C for 120 min with or without 0.05 U/ml of chondroitin ABC lyase (ICN Biochemicals, Inc., Irvine, CA) and applied to a cationic nylon (Zeta-Probe) dot blot. Disaccharides generated by the enzyme digestion fail to bind to the blot and the remaining, intact GAG was assessed by scintillation counting.

Lipophilic PG. Lipophilic PG was quantified by spotting 1:100 dilutions of cell extracts on silicone-treated paper (1-PS, Whatman Inc., Clifton, NJ) circles (25 mm) pretreated by soaking for several days in 3:1 propanol:water followed by overnight soaking in 3 M sodium chloride in 10 mM Tris-HCl (pH 8.0) (Jalkanen et al., 1987). After 24 h, the filters were washed on a vacuum manifold (Hoeffer Scientific Instruments, San Francisco, CA) in 3 M sodium chloride, 10 mM Tris-HCl (pH 8.0) followed by distilled water. Under these conditions, lipophilic PG binds, whereas nonlipophilic PG (e.g., the trypsin-released ectodomain or extracellular PGs) fails to bind.

Core Protein. Relative amounts of core protein were determined by immunostaining PG immobilized on cationic nylon. The soluble PG extracted by the low pH Triton-salt method described above was purified by chromatography on DEAE-Sephacel (>90% recovery) followed by mAb 281 affinity chromatography (65–75% recovery) as described above. Dilutions of antigen in TUT, representing 4,000, 2,000, and 1,000 dpm, respectively, were applied to a dot blot consisting of a sandwich of nitrocellulose overlying cationic nylon. The PG fails to bind to nitrocellulose, but potentially contaminating antibody from the affinity column does bind and is removed. The nylon blot is then blocked overnight at 37°C in Tris-buffered (10 mM, pH 7.5) saline containing 10% FBS, 0.3% Tween 20 and 0.02% sodium azide. The core protein is detected by incubation for 2 h in saline + 1% FBS containing 50 µg/ml mAb 281, followed by five washes in saline and a second 2-h incubation in saline + 1% FBS containing a 1:200 dilution of horseradish peroxidase–conjugated goat anti-rat IgG (Jackson Immunoresearch). After five washes in saline, the blot is developed in 15 ml saline containing 0.054 ml 3% hydrogen peroxide and 3 ml chloronaphthol (Sigma Chemical Co.) dissolved at 3 mg/ml in methanol. Binding of correct dilutions of PG to the blot was confirmed by scintillation counting after the immunological analysis.

DNA Quantification

DNA was quantified as described by Kapuscinski and Skoczylas (1977). Briefly, cell extracts in TUT were diluted 1:100 in DNA assay buffer (50 mM sodium phosphate (pH 7.4), 2 M sodium chloride, 2 mM EDTA [Na_2]), combined with 1 µg Hoechst 33258 dye (Sigma Chemical Co.), and the fluorescence assessed by spectrofluorimetry. Unknowns were compared with a standard curve generated using calf thymus DNA (Sigma Chemical Co.).

Indirect Immunofluorescent Microscopy

Cells on coverslips were fixed directly for 24 h in calcium- and magnesium-containing PBS (CM-PBS) containing 4% formaldehyde for visualization of apical antigens or permeabilized by extraction in 0.5% Triton X-100, 0.15 M sodium chloride, 10 mM Tris-HCl (pH 7.5) for 10 min on ice before fixation to visualize basal antigens. Cells grown on Transwell filters were stained without permeabilization as antibody readily diffuses through the filter. Fixed monolayers were incubated for 60 min in CM-PBS containing 50 mM glycine, then incubated for 60 min at room temperature in CM-PBS containing primary and secondary antibodies, respectively, each followed by fivefold washes with CM-PBS. The coverslips or filters were mounted in Immu-Mount (Shandon) containing 1 mg/ml p-phenylenediamine as a free radical scavenger. Primary antibodies were nonspecific rat serum IgG (50 µg/ml, Jackson Immunoresearch), cell surface PG-specific mAb 281 (rat IgG_{2A}, 50 µg/ml), and rabbit anti-FN serum (1:200 dilution; kindly supplied by Dr. Deane Mosher, Department of Medicine, University of Wisconsin). Secondary antibodies were TRITC-conjugated goat anti-rat IgG and FITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, Bar Harbor, ME). Slides were viewed by epifluorescence on a Nikon Microphot-FX.

Results

Polarization-dependent Response to TGF-β

Effect on Cell Density and Morphology. Prior reports indicate that TGF-β affects the behavior of epithelial cells in vitro and in vivo, most notably inhibiting their proliferation (Masui et al., 1986; Silberstein and Daniels, 1987). A similar inhibition of proliferation was noted for the NMuMG cells in the presence of 1 ng/ml TGF-β (data not shown).

A second response of the NMuMG cells to TGF-β is in their shape, namely, a spreading and elongation of the cell in the presence of the growth factor. Similar effects have been noted for other cells (Fanger et al., 1986). In subconfluent cultures, the cells become highly spread and elongated and are often aligned in swirls (Fig. 1, A and B). Newly confluent cultures incubated in a range of TGF-β concentrations for a 24-h period remain confluent but display an increasingly spread morphology with increasing TGF-β concentrations (Fig. 1, C-F), with the most spreading occurring at concentrations of 3 and 10 ng/ml. This takes place at the expense of other cells in the monolayer which are displaced to the medium. In contrast to newly confluent cultures, however, cultures maintained at confluence for a week before treatment with TGF-β show little morphological response to the growth factor, even at concentrations of 10 ng/ml of TGF-β (compare Fig. 1, G and H) and measurements of total DNA find little or no difference in cell number.

Distribution of Cell Surface PG and FN. Immunolocalization of cell surface PG and FN demonstrates that the relative effects of TGF-β on cell morphology are mimicked by changes in the distribution or accumulation of these antigens. As shown previously, NMuMG cells in monolayers that have reached confluence begin to restrict the cell surface PG to their basolateral surface (Rapraeger et al., 1986).

Staining of fixed, nonpermeabilized monolayers with mAb 281, a monoclonal antibody specific for the core protein of the cell surface PG (Jalkanen et al., 1985), detects PG only on those cells which have yet to completely polarize the antigen; cells that sequester the PG at their basolateral surfaces are also tightly sealed to one another and prevent antibody penetration to their lateral and basal sides.

Newly Confluent Monolayers

In monolayers that have been confluent for a day, many groups of cells display apical PG (Fig. 2 A) but may be bordered by more highly polarized neighbors that do not stain. In similar monolayers that have been extracted with Triton X-100 to allow antibody access to the basolateral surface, PG is seen on all of the cells (not shown; cf. Rapraeger et al., 1986) and FN can be detected in a scarce, patchy distribution beneath the monolayer (Fig. 2 B). Comparison of these monolayers with those treated for 24 h in TGF-β (5 ng/ml) illustrates both the cell shape change described above and a difference in the distribution of the PG and FN. The cells in TGF-β are more elongated and have greater apical surface area than their nontreated counterparts (Fig. 2 C). In addition, cells lacking apical PG are only rarely seen; rather, the mAb 281 antigen is uniformly distributed over the apical surface of almost all cells and is localized in greater abundance at the cell margins, due somewhat to staining of lateral cell surfaces. Examination at high magnification shows that staining at the margins of the cells can also be attributed to pseudopodia which often overlap onto the apical surface of adjacent cells and stain heavily for the PG. This is not seen in the nontreated cells. Staining of permeabilized monolayers shows PG on all basolateral surfaces (not shown) as noted for cells cultured in the absence of the growth factor.

Staining for FN demonstrates an enhanced accumulation of this matrix component in the presence of TGF-β (Fig. 2 D). The FN is deposited as thick, fibrous strands on the basal side of the monolayer, defined by differential focussing at high magnification. In contrast to the relatively uniform distribution of the cell surface PG, these strands underlie a fraction of the cells in the monolayer and occasionally encircle the basolateral surface of a single cell.

Postconfluent, Polarized Monolayers

Immunostaining shows that TGF-β treatment of postconfluent monolayers on plastic fails to affect the PG distribution and FN accumulation of the majority of these cells; the cells are polarized with respect to the PG, which is not confined to their basolateral cell surface, and do not accumulate a noticeable FN matrix (not shown). However, postconfluent cells do respond, using FN accumulation as a marker, if cultured on permeable filters. Cells plated on Transwell filters and cultured for 7 d postconfluence were treated for 24 h with TGF-β, which was supplied to either the apical or basal medium compartment of these filter chambers. Polarization of the NMuMG cells before treatment was confirmed by the sequestration of cell surface PG at the basolateral cell surface (not shown). As on plastic, the monolayers also appear sealed to the penetration of antibodies, as mAb 281 added to the apical medium compartment fails to stain the basolateral PG (not shown). Cells cultured in the absence of TGF-β (Fig.
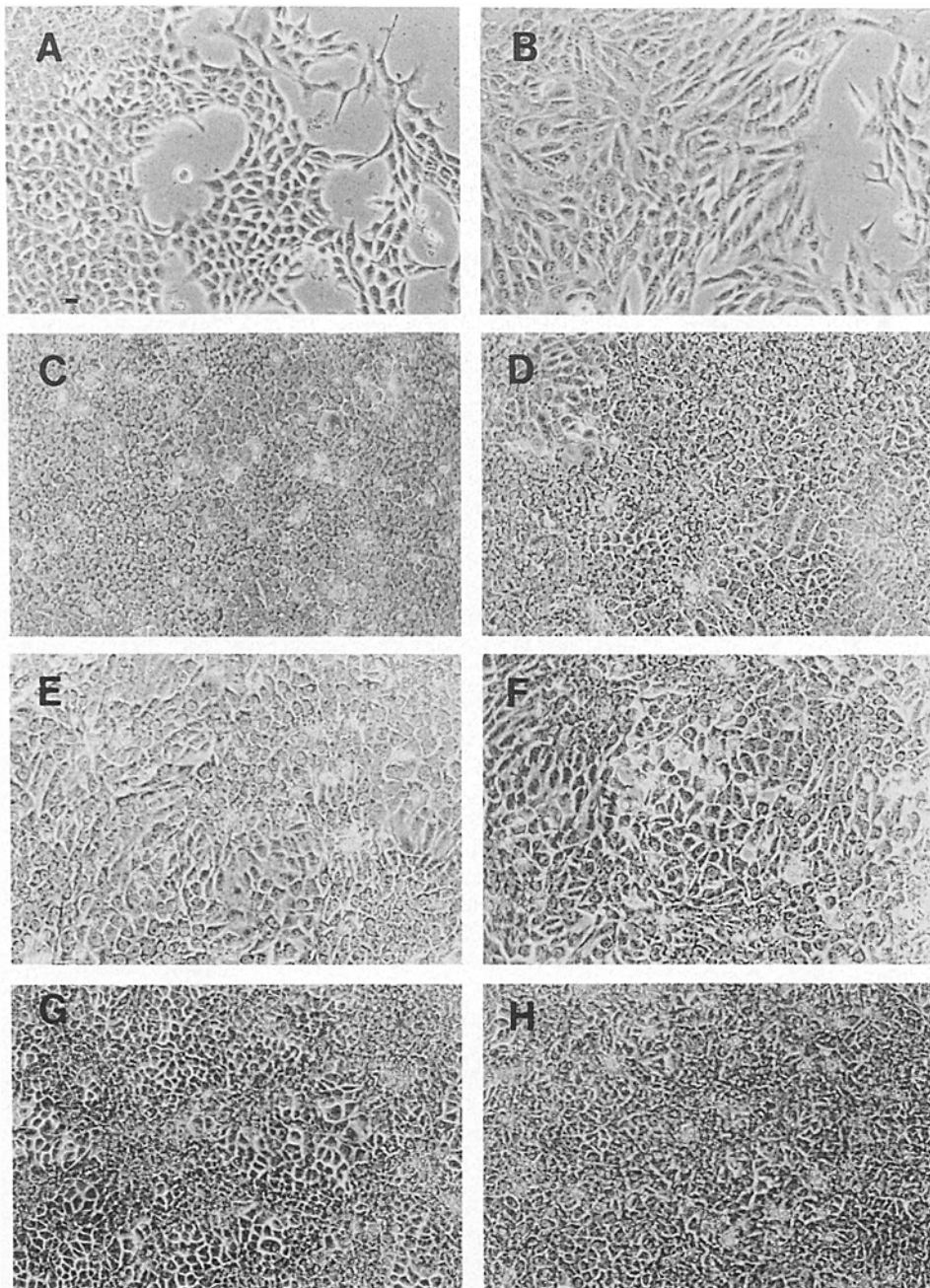
Figure 1. Effect of TGF-β on mammary epithelial cell morphology: dependence on cell density. NMuMG cells were plated either at 10% of a confluent density and allowed to proliferate for 3 d to generate a "subconfluent" monolayer, or at 25% of confluence and allowed to proliferate 3 d to generate a "newly confluent" monolayer and for 10 d to generate a "postconfluent" monolayer. TGF-β_1 was then added at varying concentrations and the cell morphology was examined following 24 h. (*A*) subconfluent, no TGF-β; (*B*) subconfluent, 10 ng/ml TGF-β; (*C*) newly confluent, no TGF-β; (*D*) newly confluent, 1 ng/ml TGF-β; (*E*) newly confluent, 3 ng/ml TGF-β; (*F*) newly confluent, 10 ng/ml TGF-β; (*G*) postconfluent, no TGF-β; (*H*) postconfluent, 10 ng/ml TGF-β. Bar, 10 μm.

3 *A*) failed to accumulate a FN matrix. The same result was obtained if the factor (5 ng/ml) was present only at their apical surfaces (Fig. 3 *B*). However, addition of 5 ng/ml TGF-β to the basal medium compartment, thus permitting access to the basolateral cell surface, resulted in the accumulation of a FN matrix (Fig. 3 *C*). Thus, the ability of polarized cells to respond is correlated with the availability of TGF-β at their cell surfaces, rather than their state of polarization per se.

Altered Expression of Cell Surface PG in TGF-β

Effective Concentration of TGF-β. NMuMG mouse mammary epithelial cells incorporate $^{35}SO_4$ mostly into GAG,

which on these cells is found primarily as a cell surface PG (Rapraeger and Bernfield, 1985). Quantification of $^{35}SO_4$-labeled cell surface PG on the NMuMG cells can be done in several ways, each using the distinct antigenic and biochemical properties of the PG. Initially, lipophilic (e.g., integral membrane) PG present in cell extracts was quantified in a hydrophobic blot assay.

Radiosulfate incorporation into lipophilic PG was examined in TGF-β_1 concentrations ranging from 0.3–10 ng/ml. These studies used cells just reaching confluence. Additions of TGF-β from 0.3 ng/ml to 10 ng/ml caused more than a twofold increase in incorporation of $^{35}SO_4$ into lipophilic PG, with maximal stimulation at 3 ng/ml (Fig. 4). This in-
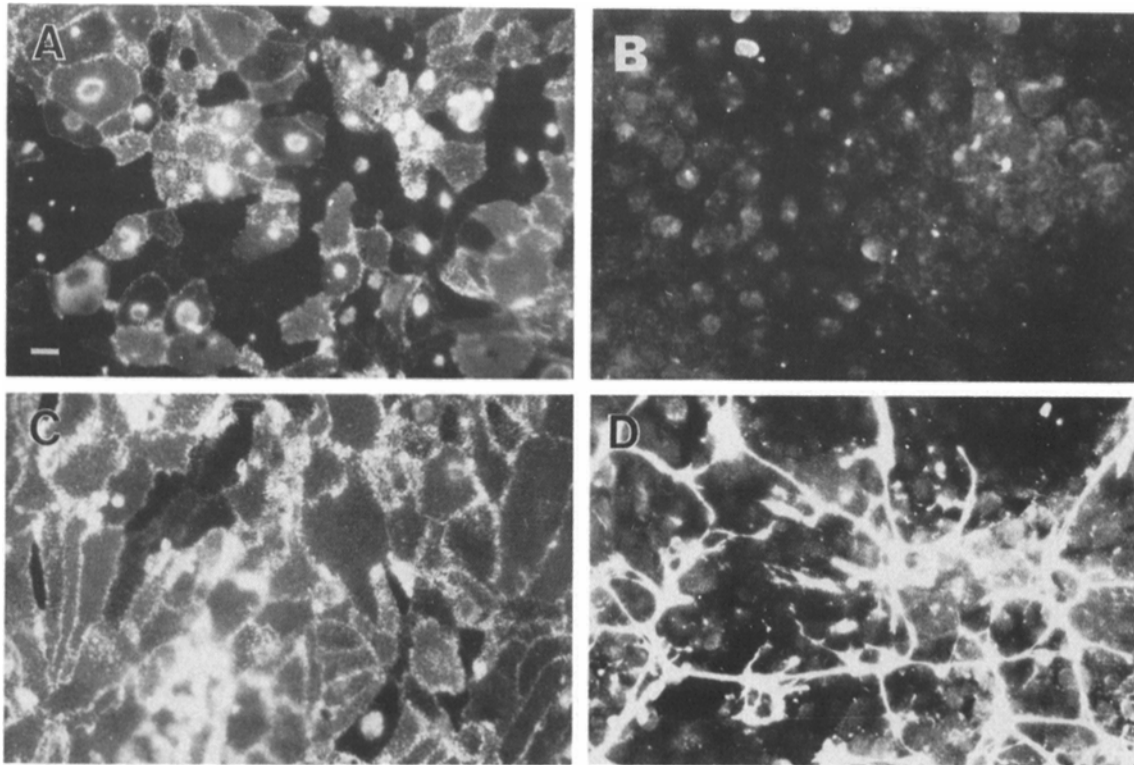
Figure 2. Localization of cell surface PG and FN in newly confluent cultures following treatment with or without TGF-β. Newly confluent (see legend to Fig. 1) NMuMG monolayers were cultured for 24 h either in the absence (*A* and *B*) or the presence (*C* and *D*) of TGF-β₁ (2 ng/ml). Monolayers were then either fixed directly and stained for cell surface PG using mAb 281 (*A* and *C*) or extracted with Triton X-100 followed by fixation and staining with anti–FN IgG (*B* and *D*). The antigens were localized by indirect immunofluorescent microscopy. Prior investigations show that some cells in newly confluent monolayers are polarized with respect to the cell surface PG and therefore fail to stain without prior permeabilization, whereas adjacent cells which have not polarized do stain (Rapraeger et al., 1986). The permeabilized cells in *B* and *D* all show basolateral stain for the cell surface PG (not shown). Bar, 10 μm.

creased accumulation of ³⁵SO₄ into lipophilic PG suggests that cell surface GAG is increased on these cells. This was corroborated by quantifying ³⁵SO₄-labeled PG released by trypsin from the surface of suspended cells. Radiosulfate incorporation into cell surface PG increases with TGF-β concentration, paralleling the increase in lipophilic PG. Identical effects were seen using porcine TGF-β₂ (not shown). The cell residue, devoid of cell surface PG, shows a 1.4–fold increase (data not shown). PG in the culture medium was increased as well, but interpretation of this was difficult due to the variable numbers of floating cells that accumulate during a long labeling period and it was not examined further.

Cells cultured 1 wk postconfluence on Transwell filters also showed maximal incorporation of radiosulfate into cell surface GAG at 3 ng/ml of TGF-β. This increase ranged from 2.5- to 3-fold and diminished at 10 ng/ml (data not shown). Postconfluent cells cultured on plastic failed to reach maximal stimulation even at 10 ng/ml.

Increased Synthesis of ³⁵SO₄-labeled GAG. To assess accumulation of radiolabeled cell surface PG, cells were preincubated in TGF-β (2 ng/ml) for 3 h, then incubated in the growth factor and radiosulfate for up to 48 h. To examine initial synthesis, several time points were taken during the first 8 h. Only slight differences in accumulation were seen in the culture medium or in the "extracellular PG" compartment,

defined as the PG remaining in a soluble form when the cells are initially scraped and sedimented, in either the presence or absence of TGF-β (not shown). However, radiolabeled GAG appearance at the cell surface (Fig. 5) was enhanced in TGF-β beginning 2–4 h after radiosulfate addition (or 5–7 h of TGF-β treatment) and continued to increase even up to 48 h, where a ∿2.5–fold enhancement was seen (Fig. 5). The intracellular compartment showed a ∿1.4–fold increase that remained constant in amount during the labeling period and may reflect an augmented amount of precursor or lysosomal PG (not shown).

Increased accumulation of radiolabeled PG at the cell surface may be due to an increased rate of synthesis or to a decreased rate of degradation. However, monitoring the disappearance of cell surface PG during a chase fails to detect an effect of TGF-β on the PG degradation rate (Fig. 6). After a 24-h label, cells treated with TGF-β have accumulated twofold more cell surface label than nontreated cells. Continued incubation of these cells in the absence of radiosulfate demonstrates that the cell surface PG disappears with a half-life of ∿6 h irrespective of the presence of the growth factor. This suggests that the increased accumulation of cell surface PG occurs via augmented synthesis rather than a lowered degradation rate.
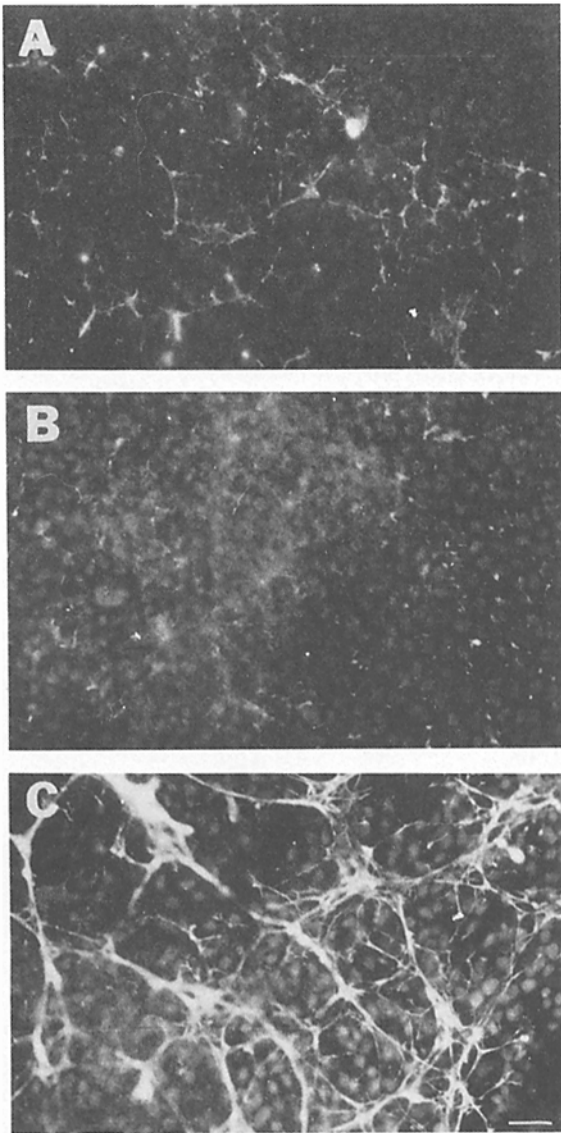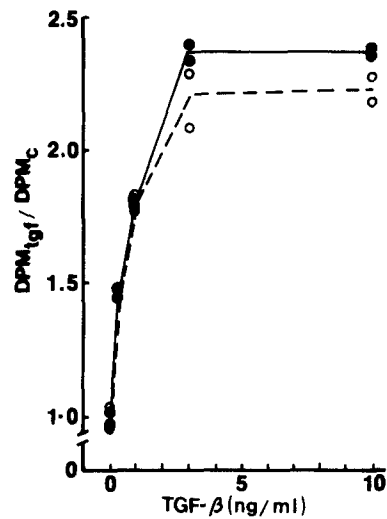
Figure 4. Quantification of $^{35}SO_4$-labeled cell surface PG vs. TGF-β concentration. Suspended NMuMG cells were extracted after a 24-h incorporation of $^{35}SO_4$ in the presence of a range of TGF-$β_1$ concentrations. DNA content was determined by a Hoechst dye assay and amount of radiolabel in lipophilic PG (●) was assessed using a hydrophobic blot assay as described in Materials and Methods. Alternatively, PG at the cell surface of suspended cells (O) was released by mild treatment with trypsin and quantified on cationic nylon blots. Data are expressed as a ratio of the incorporation in TGF-β (DPM_{tgf}) to that in the control (DPM_c).

Figure 3. Accumulation of FN by cells grown in filter chambers. NMuMG cells were grown at confluence for 1 wk on Transwell (Costar) filters, then cultured an additional 24 h without added growth factor (A), with TGF-$β_1$ (5 ng/ml) in the apical medium compartment (B), or in the basal medium compartment (C). Volumes in the apical and basal medium compartments were equal. The monolayers were then fixed and stained for indirect immunofluorescent localization of FN. Bar, 20 μm.

nylon in a series of dilutions (Fig. 7). Immunostaining of the blot with mAb 281, to quantify core protein, shows roughly twofold less reactivity in the fraction derived from TGF-β-treated cells. This estimate suggests about a twofold greater incorporation of radiolabel per core protein in the presence of TGF-β, which may account for most or all of the increased label seen on cell surfaces in the presence of the growth factor.

Increased Proportion of Chondroitin Sulfate GAG

As the cell surface PG bears not only heparan sulfate but also a minor contribution of chondroitin sulfate, the possibility that the proportion of chondroitin sulfate is altered by TGF-β
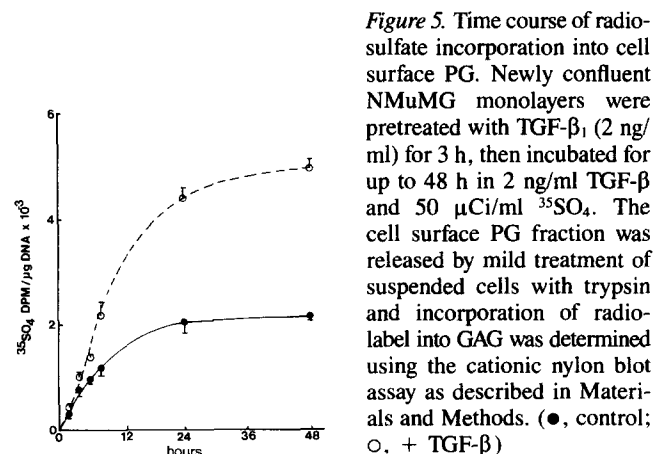
Increased ^{35}S-labeled GAG Per Core Protein

Augmented $^{35}SO_4$ incorporation into PG at the cell surface may be due either to more PGs or to more incorporation per PG. In an attempt to choose between these possibilities, cell surface PG from nontreated or TGF-β-treated cells was purified and analyzed in an immunodot assay in which PG core protein is detected by mAb 281. This assay uses cationic nylon as a blot, which quantitatively binds intact PG but binds denuded core protein only poorly (Rapraeger et al., 1985).

Equal radioactive amounts of purified $^{35}SO_4$-PG from cells treated with or without TGF-β were bound to cationic

Figure 5. Time course of radiosulfate incorporation into cell surface PG. Newly confluent NMuMG monolayers were pretreated with TGF-$β_1$ (2 ng/ml) for 3 h, then incubated for up to 48 h in 2 ng/ml TGF-β and 50 μCi/ml $^{35}SO_4$. The cell surface PG fraction was released by mild treatment of suspended cells with trypsin and incorporation of radiolabel into GAG was determined using the cationic nylon blot assay as described in Materials and Methods. (●, control; O, + TGF-β)
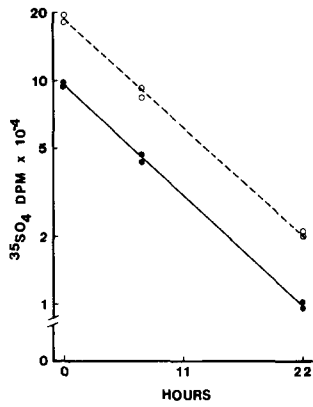
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Figure 6. Loss of $^{35}SO_4$-labeled cell surface PG from cells incubated in the presence of TGF-β. Newly confluent NMuMG monolayers were labeled for 24 h with 50 μCi/ml $^{35}SO_4$ in the presence or absence of TGF-β₁ (2 ng/ml) and then chased without radiosulfate and unchanged TGF-β treatment for up to 22 h. Cell surface PG was quantified as in Fig. 5. The data are normalized for differences in DNA and expressed as dpm remaining per 35-mm dish. (●, control; ○, + TGF-β)

Figure 8. Ion exchange chromatography of cell surface PG derived from cells treated with or without TGF-β. The $^{35}SO_4$-labeled ectodomain of the cell surface PG, derived by mild trypsin treatment of newly confluent cells cultured for 24 h with or without 5 ng/ml TGF-β₁, was bound to DEAE-Sephacel and eluted in a sodium chloride gradient ranging from 0.2 to 1.0 M. Radiolabeled PG (●) in the fractions was assessed by binding to cationic nylon blots. The amount and distribution of chondroitin sulfate in the chromatogram (stippled area) was determined by digestion with chondroitin ABC lyase. The disaccharides generated by this digestion fail to bind to the blot, whereas the PG still bearing heparan sulfate chains (confirmed by nitrous acid digestion) is quantitatively retained (○).

treatment was examined. The ectodomain of the PG was harvested by mild treatment of $^{35}SO_4$-labeled NMuMG cells with trypsin, isolated by mAb 281 affinity chromatography, and examined on ion exchange chromatography. The trypsin-released cell surface PG from TGF-β-treated cells (Fig. 8 *B*) behaves differently when compared to PG derived from nontreated cells (Fig. 8 *A*). It elutes as a broader peak, primarily due to a trailing edge. As the binding of the PG to the column is via its complement of GAG chains, with chondroitin sulfate binding more strongly than heparan sulfate, this result suggests that the PG in the trailing edge of the peak may be richer in chondroitin sulfate. This was verified by enzymatic analysis. Treatment of the eluted fractions with chondroitin ABC lyase demonstrates that a small proportion of the radiolabel from nontreated cells is in chondroitin sulfate (Fig. 8 *A*, stippled area). The remainder is heparan sulfate, defined by its susceptibility to nitrous acid or heparitinase. Displacement of the chondroitin sulfate profile to a slightly higher salt elution point than the heparan sulfate probably reflects variability in the actual number of each type of chain per PG molecule, as reported previously (Rapraeger et al., 1985).

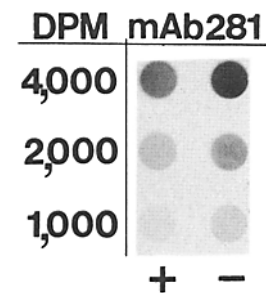In response to TGF-β treatment, the proportion of radiosulfate in chondroitin sulfate is increased. Although the

majority of the radiolabel remains as heparan sulfate, PG molecules eluting at the trailing edge of the profile contain increasing proportions of radiolabel in chondroitin sulfate (stippled area, Fig. 8 *B*), reaching a maximum of ~40% of the radiolabel.

Discussion

Transforming growth factor-β promotes a wide array of cell-type specific behavior, including effects on cell proliferation and differentiation (reviewed by Massagué, 1987) and the accumulation of ECM components, such as FN and type I collagen, and their receptors (Ignotz and Massagué, 1987). Therefore, we were interested in studying the effect of TGF-β on the expression of cell surface PG by mouse mammary epithelial cells. Treatment with TGF-β causes enhanced radiosulfate incorporation into lipophilic (integral membrane) PG, with an increase of ~2.5-fold at 3 ng/ml. The augmented radiolabel is (*a*) displayed as PG at the cell surface, (*b*) due to an increase in synthesis, not an alteration in the degradation rate of the PG, and (*c*) mostly, if not completely, attributed to an alteration in the GAG complement of the PG. At a minimum, this alteration involves a change in the proportion of chondroitin sulfate to heparan sulfate attached to the core protein of this novel hybrid proteoglycan.

Effective Concentration of TGF-β

The effective concentration of TGF-β required to maximally affect the NMuMG cells is 3 ng/ml. Similar concentrations affect matrix accumulation by NRK-49F fibroblasts and other cell lines (Ignotz et al., 1987; Bassols and Massagué, 1988). However, a 100-fold lower concentration affects the proliferation of normal human bronchial epithelial cells

Figure 7. Incorporation of radiosulfate per cell surface core protein in the presence or absence of TGF-β. Newly confluent NMuMG monolayers were labeled with radiosulfate for 24 h with or without treatment with TGF-β₁ (5 ng/ml) and extracted by treatment with low pH Triton/salt and purified on DEAE-Sephacel, selective for the GAG chains on the PG, followed by mAb 281 affinity chromatography, which is selective for the PG core protein. Serial dilutions of radioactive PG (4,000, 2,000, and 1,000 dpm) from cells cultured in the absence (−) or presence (+) of TGF-β were spotted on cationic nylon. Relative amounts of PG core protein were then assessed by an antibody-coupled peroxidase reaction using mAb 281. When compared to the nontreated cells, cells treated with TGF-β have twice as much label in GAG for an equivalent amount of core protein.
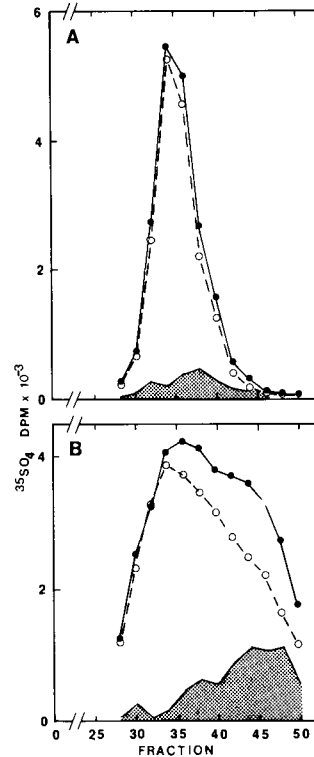
(Masui et al., 1986). A complicating factor in determining the effective concentration was the reduction in response of confluent monolayers grown on plastic. This appears to be temporally related to their polarization of the cell surface PG and likely other basolateral membrane proteins. An apparent explanation for this is that the TGF-β receptor is present largely on the basolateral cell surface, as polarized cells do respond to TGF-β if cultured on permeable filter supports, which allow diffusion of the growth factor from the culture medium to the basolateral cell surface. Direct identification of the receptor will determine whether this is because the basolateral surface represents the majority of the cell surface area or because the receptor is actually absent from the apical cell surface.

TGF-β binds at least three cell surface receptors (Cheifetz et al., 1987; Segarini et al., 1987) and their relative affinities and activities when occupied by TGF-β_1 or TGF-β_2 remain to be completely understood. However, augmented chondroitin sulfate PG, FN, and collagen production are reportedly mediated via the ~250-300 kD (type III) receptor (Ignotz and Massagué, 1987; Bassols and Massagué, 1988) based primarily on the finding (Cheifetz et al., 1987) that this receptor, in contrast to the other two, binds TGF-β_1 and β_2 with equal affinities. As the NMuMG cells deposit FN and appear to respond equally to both TGF-β homodimers, this is consistent with a response mediated via the type III receptor. Interestingly, during review of this report, the 250-kD receptor on Swiss 3T3 cells has been identified as a heparan sulfate/chondroitin sulfate hybrid PG (Segarini and Seyedin, 1988) that binds TGF-β via its core protein. The presence of the type III receptor in NMuMG cells and the potential relationship between that receptor and this epithelial cell surface PG remains to be examined. However, identification of this or a related PG as the responsive TGF-β receptor would explain the polarization-dependent response of these cells and pose questions regarding changes in its structure affecting its activity.

Alteration of the Cell Surface Proteoglycan During TGF-β Treatment

The NMuMG cell surface PG is a complex macromolecule that bears heparan sulfate GAG and a complement of chondroitin sulfate GAG, suggesting a regulatory mechanism. The effect of TGF-β on the NMuMG cells may reflect this regulation; e.g., promote chondroitin sulfate synthesis on the epithelial PG core protein.

The relative amounts of core protein on treated and non-treated cells are similar as determined by mAb 281 recognition. Although it is possible that the core protein from TGF-β treated cells might not be recognized by the antibody due to a change in the attachment of GAG chains, perhaps blocking the antigenic site, this is unlikely as the PG is isolated by mAb 281 affinity chromatography. This suggests that the additional radiolabel appearing at the cell surface is incorporated as more chondroitin sulfate GAG onto the core protein that normally contains primarily heparan sulfate. This may be regulated either by the addition of more chondroitin sulfate chains, perhaps at the expense of heparan sulfate, or by an increase in the size of the chains. The chondroitin sulfate and heparan sulfate chains do appear longer, but not by enough to account for the entire increase (Rasmussen, S., and A. Rapraeger, unpublished observations).

Whereas this report examines the modification of a heparan sulfate cell surface PG by the addition of chondroitin sulfate, the effect of TGF-β on the synthesis of chondroitin sulfate PGs has been reported by other workers. Chondroitin sulfate is increased in the culture medium and cell layer of smooth muscle cells (Chen and McKeehan, 1987). Most recently, Bassols and Massagué (1988) have reported increased synthesis of chondroitin sulfate PGs in the matrix and culture medium of several cell lines, including mink lung epithelium. These increases are several-fold greater than that reported here and include augmented core protein synthesis as well as an increase in the length of the chondroitin sulfate chains. Whether heparan sulfate PG synthesis is refractory to TGF-β in all cells, and whether this explains the absence of elevated core protein accumulation in the NMuMG cells is not clear.

Changing the kind, number or character of the GAG chains attached to the core protein of the NMuMG cell surface PG may have profound effects on its potential function as a receptor. Where studied, the binding of matrix components by the PG has been dependent on its GAG chains, primarily the heparan sulfate chains which bind to interstitial collagens (Koda et al., 1985), FN (Saunders and Bernfield, 1988), and thrombospondin (Sun, X., D. Mosher, and A. Rapraeger, manuscript submitted for publication). A change in the number or sulfation of the heparan sulfate chains, or a change in the number and orientation of the chondroitin sulfate chains may affect this binding, thereby affecting its receptor role. The degree to which the alteration in PG structure is involved in the multiple effects of TGF-β is currently being studied.

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