Decreased Type II/Type I TGF-β Receptor Ratio in Cells Derived from Human Atherosclerotic Lesions

Conversion from an Antiproliferative to Profibrotic Response to TGF-β1

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Abstract

Atherosclerosis and postangioplasty restenosis may result from abnormal wound healing. The present studies report that normal human smooth muscle cells are growth inhibited by TGF-β1, a potent wound healing agent, and show little induction of collagen synthesis to TGF-β1, yet cells grown from human vascular lesions are growth stimulated by TGF-β1 and markedly increase collagen synthesis. Both cell types increase plasminogen activator inhibitor-1 production, switch actin phenotypes in response to TGF-β1, and produce similar levels of TGF-β activity. Membrane cross-linking of ^{125}I-TGF-β1 indicates that normal human smooth muscle cells express type I, II, and III receptors. The type II receptor is strikingly decreased in lesion cells, with little change in the type I or III receptors. RT-PCR confirmed that the type II TGF-β1 receptor mRNA is reduced in lesion cells. Transfection of the type II receptor into lesion cells restores the growth inhibitory response to TGF-β1, implying that signaling remains responsive. Because TGF-β1 is overexpressed in fibroproliferative vascular lesions, receptor-variant cells would be allowed to grow in a slow, but uncontrolled fashion, while overproducing extracellular matrix components. This TGF-β1 receptor dysfunction may be relevant for atherosclerosis, restenosis, and related fibroproliferative diseases. (*J. Clin. Invest.* 1995. 96:2667–2675.) Key words: cell proliferation • extracellular matrix • transforming growth factor-β1 receptors • atherosclerosis • restenosis

Introduction

The prevailing theory proposes that atherosclerosis and postangioplasty restenosis result from aberrant wound healing processes (1). Because the excessive fibroproliferative response occurs within the lumen of coronary, carotid, and peripheral arteries in 30–40% of patients undergoing angioplasty or vascular surgery, restenosis remains a major clinical problem. Both atherosclerotic and restenotic lesions show remarkably low rates of cell proliferation (2, 3), consistent with the months to years required before morbidity. The restenotic lesion is composed largely (> 80%) of cells expressing antigenic markers consistent with a myofibroblast or a smooth muscle cell (SMC).[1] While these cells grow quite slowly, they produce and accumulate an excess of extracellular matrix proteins, as shown both by histology and their behavior in vitro (3, 4).

Transforming growth factor-β1 (TGF-β1), in particular, has very potent effects upon each of the known components of wound repair (5, 6). TGF-β1's relatively unique ability to both positively and negatively regulate the components of inflammation and wound repair implies that it may be vital in orchestrating these complex processes (7). The importance of TGF-β1 in the vascular repair process has been established by several lines of evidence. TGF-β1 mRNA and immunoreactivity are increased in human restenotic lesions (8) and in animal models after vascular injury (9), after induced hypercholesterolemia in primates (10), and after DOC/salt hypertension (11). The responsiveness of vascular cells to TGF-β1 is demonstrated by in vitro studies (12, 13) and in vivo data which indicate that infusions of TGF-β1 (9) or transfection of TGF-β1 cDNA (14) into injured arteries strongly accelerate lesion formation, by increasing cellularity and markedly increasing extracellular matrix accumulation. Likewise, antibodies to TGF-β1 reduce the development of vascular lesions after balloon injury in rats (15).

Cells derived from atherosclerotic and restenotic lesions appear to differ from normal vascular SMC in both their growth control and extracellular matrix synthesis. Cells grow more rapidly from restenotic lesions than from primary atherosclerotic plaques (16), and cells cultured from restenotic sites are resistant to inhibition by heparin (17), which is a well-established inhibitor in normal arterial SMC. Cells grown from vascular lesions also produce greater amounts of extracellular matrix proteins than normal SMC (3). Based on the pathology of atherosclerosis and restenosis, as well as the dysregulated growth and fibrotic behavior of the cells in vitro, the current studies examined the hypothesis that cells migrating and proliferating from vascular lesions may exhibit a different response to TGF-β1 than normal vascular SMC. The data indicate that compared with cells grown from normal arteries, cells derived from plaques are not growth inhibited by TGF-β1 and show a more elaborate fibrotic response to TGF-β1. This is associated with a change in the pattern of membrane receptors for ^{125}I-

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1. *Abbreviations used in this paper:* DSS, disuccinimidyl suberate; PAI-1, plasminogen activator inhibitor-1; RT, reverse transcriptase; SMC, smooth muscle cells; uPA, urokinase-type plasminogen activator.

TGF-β1 and is partially correctable by transfection of a type II receptor into lesion cells. The presence of a cell type in vascular lesions that exhibits a different pattern of TGF-β1 receptors and functionally different growth and fibrotic responses to TGF-β1 may have a direct bearing on the course of postinjury repair in the coronary, carotid, and peripheral arteries.

Methods

Reagents

The radiochemicals L-[5-^{3}H]-proline (28 Ci/mmol), ^{125}I-TGF-β1 (192 μCi/μg), and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were purchased from New England Nuclear (Wilmington, DE). TGF-β1 (human recombinant) was purchased from R&D Systems (Minneapolis, MN) and was kindly provided by Berlex Industries (San Francisco, CA). Disuccinimidyl suberate (DSS), a bifunctional cross-linking agent, was purchased from Pierce Chemicals (Rockford, IL).

Vascular specimens

Coronary vessel wall biopsies were obtained by directional atherectomy at The New York Hospital/Cornell Medical Center. Carotid, iliac, and femoral artery plaques were obtained from patients undergoing endarterectomy at the same institution. All specimens were waste tissue collected in accordance with a protocol approved by the institutional review board. Human SMC from normal arteries were obtained from aortic tissue of young adult accident victims (Clonetics Corp., San Diego, CA), neonatal aorta (CRL 1999; American Type Culture Collection, Rockville, MD), and from the grossly normal regions of atherosclerotic femoral arteries removed for grafting.

Cell culture

Cells from vascular lesions were obtained by finely dicing the specimens and adhering them to collagen (Vitrogen, Collagen Corp., Palo Alto, CA) or fibronectin-coated flasks in medium 199 (M199; Gibco Laboratories, Grand Island, NY) containing 15% FBS (Gemini Bioproducts, Calabasas, CA), gentamicin sulfate (50 μg/ml; Gibco), and 10% postculture media collected from prior lesion-derived cultures. Cells extending from the explants onto the flask surface were subpassaged with trypsin/EDTA (Gibco) at a 1:2 split ratio onto uncoated tissue culture flasks and grown in M199, 15% FBS, and gentamicin without postculture media. Cell cultures were obtained from primary coronary atherosclerotic lesions, restenotic coronary lesions, or from carotid or peripheral endarterectomy specimens as specified for each experiment (lesion cells). Both lesion and normal SMC were cultured in the same media (M199+15% FBS) for several weeks before use in these experiments, and they were both used within the first six passages.

DNA synthesis

The effect of TGF-β1 on the rate of DNA synthesis was examined by semiautomated methods. Cells were plated at 1×10^4 cells/well of 96-well microtiter plates at least 24 h before the assay. TGF-β1 was introduced into normal serum-containing growth media for 20 h before the cells were pulsed with [^{3}H]thymidine (1 μCi/ml) for 4 h. Cells were collected with trypsin and a cell harvester (Wallac, Inc., Gaithersburg, MD), and the DNA-incorporated label was determined by scintillation counting (Betaplate; Wallac, Inc.) (n = 6 per point).

TGF-β activity

The production of TGF-β activity by vascular cells was examined by a bioassay sensitive to TGF-β1, -β2, and -β3. Serum-free supernatants were collected from equal cell numbers of normal or lesion cells. In some cases, the supernatants were concentrated before the assay using centrifugal membranes (Amicon-10; 10K cutoff; Amicon, Inc., Beverly, MA). The supernatants were assayed for their ability to inhibit DNA synthesis in CCL64 mink lung epithelial cells (Mv1Lu; ATCC). Inhibition was compared with a standard curve of recombinant human TGF-β1. Neutralizing antibody (polyclonal anti-β1, -β2, -β3; R&D Systems) was used to neutralize TGF-β activity in selected supernatants.

Plasminogen activator inhibitor-1 activity

Conditioned media from cells incubated with TGF-β1 (0–10 ng/ml) for 48 h under serum-free conditions were tested for their ability to inhibit a standard amount of urokinase-type plasminogen activator (uPA; American Diagnostica, Greenwich, CT). Samples were preincubated with uPA (10 mU) for 30 min at 37°C before the addition of plasminogen (American Diagnostica) and the fluorometric peptide plasmin substrate d-Val-Leu-Lys-AMC (Enzyme Systems, Dublin, CA) for 2.5 h at 37°C. Cleavage of the substrate was monitored in a Fluoroskan microplate reader (Flow Laboratories, Inc., McLean, VA), and uPA activities were interpolated from a standard curve using 0.1–100 mU of uPA.

α-Actin expression

The induction of the contractile α-actin isoform by TGF-β1 in cells from atherosclerotic lesions was examined by two methods.

Immunostaining. Lesion cells were plated at 1×10^4 cells/well of an 8-chamber glass slide (LabTek) in M199 + 10% FBS. The next day the cells were changed to 1% FBS or 1% FBS containing 1.0 ng/ml TGF-β1. After 72 h, the cells were fixed with 4% formaldehyde in PBS, and the α-actin filaments were stained with a monoclonal antibody (1E4 clone; Sigma Immunochemicals, St. Louis, MO) detected with FITC-labeled goat anti-mouse IgG (Sigma).

Western blot. Cells derived from a restenotic human coronary artery were plated at 5.0×10^5 cells per 75-cm^2 flask in M199 + 10% FBS for 24 h and then changed to 1% FBS or 1% FBS + 1 ng/ml TGF-β1 for 24 h. The cells were scraped, lysed, and electrophoresed under reducing conditions on a 7.5% SDS-PAGE gel. The gel was transferred to nitrocellulose and probed with the same actin antibody for 3 h at room temperature. After stringent washing, the antibody was detected with ^{125}I-protein A and exposed to Kodak XAR film for 7 h at −70°C.

Extracellular matrix production

The induction of extracellular matrix production in normal SMC and lesion cells in response to TGF-β1 was evaluated by incorporation of [^{3}H]proline into insoluble, extracellular matrix protein, which is largely collagenous. Cells from normal arteries or atherosclerotic lesion (endarterectomy) were plated at 2.5×10^4 cells/well (4.5 cm^2) in DME (proline-free) with 1% FBS and gentamicin sulfate (50 μg/ml). After 4 h, TGF-β1 and [^{3}H]proline (4 μCi/well) were added for an additional 72 h. The supernatant was then collected, centrifuged (2,000 $g \times 10$ min) to remove cells, and precipitated with a final concentration of 10% TCA. After centrifugation (12,000 $g \times 10$ min), the protein pellet was counted to evaluate incorporation into nonmatrix, soluble proteins. The adherent cell monolayer was washed with PBS twice and then dissolved with 1 M NaOH for 30 min at 37°C, and then counted to evaluate cell-associated incorporation.

^{125}I-TGF-β1 binding and cross-linking

The pattern of membrane receptors for TGF-β1 was examined by chemically cross-linking ^{125}I-TGF-β1 to the cell surface followed by SDS-PAGE and autoradiography. Lesion cells or normal human SMC were plated at 1×10^5 cells/well (9.6 cm^2) in normal growth media for 24 h before changing the cells to serum-free M199 for 24 h. Cells were changed to fresh serum-free media 1 h before applying ^{125}I-TGF-β1 (1 ng/ml/well) in binding media (M199 with 0.05% gelatin) at 4°C with or without a 50-fold excess of unlabeled TGF-β1. After a 3-h binding period, DSS was added to the wells at a final concentration of 1 mM for 15 min. The cells were washed with binding media, scraped into detachment buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 0.3 mM PMSF), and centrifuged at 12,000 g for 2 min. The cells were resuspended in SDS sample buffer with β-mercaptoethanol for 30 min at 37°C, centrifuged, and the dissolved proteins were separated on a 7.5% SDS-PAGE gel. Molecular weights were determined from ^{14}C-labeled markers (CFA-626; Amersham Corp., Arlington Heights, IL). The dried gel was exposed to Kodak XAR film and the gel was additionally analyzed with a PhosphoImager (Molecular Devices, Menlo Park, CA).

Reverse transcriptase (RT)-PCR of receptor mRNA

Total RNA was prepared, using the Tri-Reagent as described by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH), from normal or lesion cells under the conditions similar to those used for receptor cross-linking, but using larger cell numbers ($0.5-1.0 \times 10^6$ cells/75 cm^2). Total RNA (1 μg) was reverse transcribed with Mmlv reverse transcriptase at 42°C with random hexamer primers (Perkin-Elmer Corp., Norwalk, CT) followed by 35 cycles of PCR with *Taq* polymerase at annealing temperature of 57°C with primer extension at 72°C and 2.5 mM MgCl, pH 8.0. PCR primers were designed using MacVector (Kodak, Rochester, NY). The type II receptor was amplified using PCR primers (5′-TGCACATCGTCCTGTGGAC-3′; 5′-GTCTCAAACTGCTCTGAAGTGTTC-3′) annealing to bases 370–388 (+strand) and 1153–1131 (−strand), respectively, of the human type II TGF-β receptor (reference 18; GenBank No. M85079). The type I receptor was amplified using primers (5′-CGTGCTGACATCTATGCAAT-3′; 5′-AGCTGCTCCATTGGCATAC-3′) annealing to bases 1268–1287 (+strand) and 1519–1501 (−strand), respectively, of the human type I receptor (ALK5 clone, reference 19; GenBank No. L11695). TGF-β1 was amplified with primers (5′-TCCGCAAGGACCTCGGCTGGA-3′; 5′-ATCATGTTGGACAGCTGCTCC-3′) annealing to bases 1749–1769 (+) and 1992–1972 (−) of the human sequence (reference 20; GenBank No. X02812). The identity of the type I and II receptor PCR products was confirmed both by hybridization with radiolabeled cDNA (random primed ^{35}S-ATP) and by dideoxy sequencing (Sequenase 2.0; Amersham Corp.) of selected PCR products subcloned into PCR II (In vitrogen, San Diego, CA). The size of the amplified bands was determined by comparison with an adjacent lane containing HaeIII-digest fragments of known size of ΦX174 RF DNA (Gibco).

Transfection of receptors

The type II TGF-β1 receptor cDNA in pcDNA 1 expression vector (clone H2-3FF, reference 18, kindly provided by Dr. Herbert Lin, Whitehead Institute, Cambridge, MA) was expressed in endarterectomy-derived cells using lipid-mediated transfection. The expression vector was preincubated with LipofectAmine (Gibco) and then exposed to cells in serum-free media for 24 h. The cells were changed to fresh media containing serum with or without TGF-β1 (1 ng/ml). After an 18-h exposure, the cells were pulsed with [^{3}H]thymidine for 4 h and harvested as described.

Transfection efficiency. The percentage of lesion cells transfected by this method was evaluated with pcDNA3 (In vitrogen), which uses the same cytomegalovirus promoter, bearing a lacZ reporter gene (kindly provided by Dr. Shahin Rafii, Cornell University Medical College). After LipofectAmine transfection, the number of transfected cells was evaluated in four separate wells by overnight detection of the lacZ protein using the X-gal reagent and counting the number of blue cells.

Results

Effect of TGF-β1 on cell proliferation in normal and lesion-derived cells

Prior studies on normal SMC indicate that in human, bovine, porcine, rat, and rabbit SMC TGF-β1 is a potent inhibitor of DNA synthesis (12, 21–23), with growth arrest occurring in the late G_1 phase (22). Under specific culture conditions, and in SMC derived from aged rats (23), however, TGF-β1 can stimulate DNA synthesis (12) or increase the response to other mitogens (12, 24).

DNA synthesis. We examined the response of lesion-derived cells and observed that it differed strikingly from the response of normal human SMC. The rate of DNA synthesis in normal SMC was potently inhibited by TGF-β1 by as little as 0.01 ng/ml, reaching maximal inhibition of ~ 50–90% of the stimulated rate at 0.1–1 ng/ml (Fig. 1, *left*). In contrast, all doses of TGF-β1 stimulated DNA synthesis in lesion-derived cells. Prior studies demonstrated that DNA synthesis in lesion-derived cells was inhibited by up to 90% by inhibitors of eIF-5A hydroxylation, proving that these cells are capable of late G_1 growth arrest (4).

Because the lesion-derived lines tended to be from the coronary or peripheral arteries of elderly patients, while the normal lines tended to be from the aorta of younger accident victims, we isolated cells from the atherosclerotic lesion and the adjacent normal artery of the same patient. Cells derived from the atherosclerotic plaque were slightly stimulated at low concentrations and were not inhibited at higher concentrations (Fig. 1, *right*). Cells from the adjacent, grossly normal artery were inhibited 40% by TGF-β1 (1 ng/ml), though they appeared less sensitive than cells derived from young aortas (Fig. 1, *left*).

The results shown are representative of our experience with more than 29 separate studies on 15 different lesion-derived cell lines from coronary and peripheral vascular lesions, compared with 8 separate normal SMC lines. Of the 15 lesion-derived cell lines, 8 were derived from coronary atherectomies (4 de novo, 4 restenotic lesions), and 7 were derived from carotid or femoral/iliac endarterectomies. Only 1 of the 15 lesion-derived lines showed inhibition to TGF-β1, while all 8 normal SMC lines were sensitive to TGF-β1.

Baseline rates of [^{3}H]thymidine incorporation differed widely between cell lines but did not predict the response to TGF-β1. In Fig. 1, baseline incorporation ranged from 128±5.8 to 250±13.1 cpm/well for lesion cells and 562±65 to 3,564±139 cpm for normal cells (*left*). Thus, despite the sixfold differences in baseline rate of incorporation, both normal SMC cell lines were potently inhibited by TGF-β1. In other studies, lesion cells which were resistant to TGF-β1 had baseline rates only 50% lower than normal cells. Cell counting of ongoing cultures, in the absence of exogenously added TGF-β1, indicated that lesion cells proliferated about as well as normal cells, suggesting that the difference in baseline [^{3}H]thymidine incorporation may be due to differences in [^{3}H]thymidine uptake, which would affect the specific activity of the labeled DNA.

Cell proliferation. The effect of TGF-β1 on the short-term rate of DNA synthesis probably measures only one aspect of TGF-β1's effect upon cell proliferation over time. Thus, the effect of TGF-β1 on cell growth was examined over a 6-d period, with the addition of fresh TGF-β1 at 3-d intervals. The results corroborated the effect observed with DNA synthesis. Normal cells were inhibited by 27% ($P < 0.001$, $n = 3$) by TGF-β1 (1 ng/ml) over the 6-day period while the growth of coronary lesion-derived cells was unaffected (97% of control, P = NS). The difference between the 50–90% inhibition of DNA synthesis and the 27% inhibition of cell proliferation in normal SMC may be accounted for by the effect of TGF-β on the production of mitogens and extracellular matrix which would tend to offset the inhibitory action of TGF-β1.

Production of TGF-β1 by vascular cells

Prior evidence has established that normal human vascular SMC produce active TGF-β1 in culture (25). The production of TGF-β activity in serum-free supernatants of cells from lesions was compared with cells from normal arteries and no major differences in the production of the active or total latent activity were observed in a sensitive bioassay which responds to each of the mammalian TGF-β isoforms (β1, β2, and β3). Normal SMC produced ~ 0.22 ng/ml (per 5×10^5 cells/24 h) of active TGF-β which was ~ 20% of total, acid-activatable TGF-β (1.3 ng/ml) and comparable with that produced by lesion cells under identical conditions (0.25 ng/ml active; 1.1 ng/ml total).

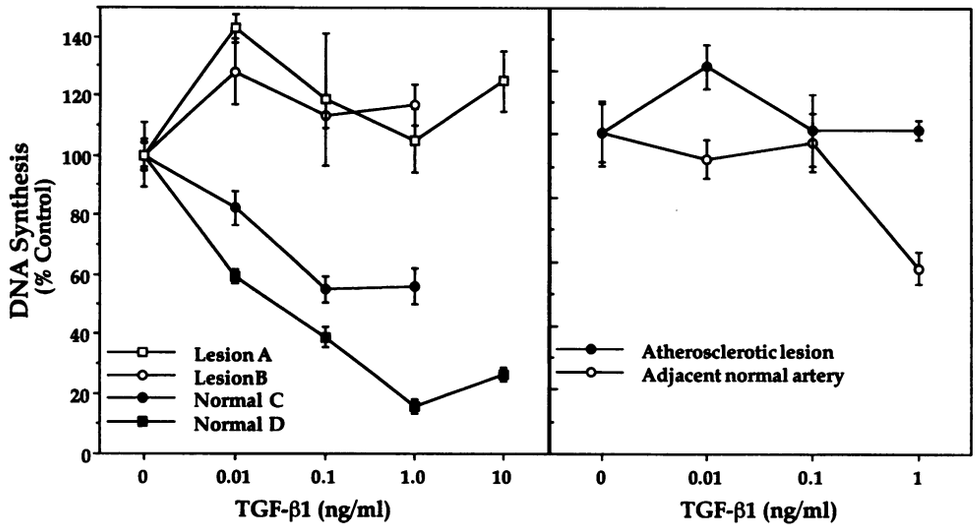

Figure 1. The effect of TGF-β1 on DNA synthesis in normal human SMC and human vascular lesion-derived cells. (*Left*) Lesion cells were grown from a coronary artery biopsy (*Lesion B*, patient 172) taken by percutaneous atherectomy of a restenotic lesion in the left anterior descending artery of a 62-yr-old male patient, and from a superficial femoral artery lesion (*Lesion A*, patient 3) of a 66-yr-old female acquired by surgical endarterectomy. Normal SMC were acquired from the aorta of two different young healthy accident victims. Both cell lines were plated at 2×10^4 cells/well of a 96-well microtiter plate for 48 h before exposure to TGF-β1 in growth media containing 15% FBS. After 20 h, [^{3}H]thymidine was added for 4 h and then the DNA was collected by semiautomated techniques and counted in a scintillation counter. Matching symbols (i.e., *open* and *filled circles*) were assayed in the same experiment. Points reflect the mean±SEM of four wells per group expressed as a percentage of the control (no TGF-β1) for that cell line. (*Right*) Cells were subcultured, under identical conditions, from the atherosclerotic lesion or the adjacent, underlying medial portion of a human femoral artery of the same patient. The response to TGF-β1 was evaluated as above.

Induction of the contractile α-actin isoform in lesion cells by TGF-β1

An important aspect of wound healing is wound closure, facilitated by the induction of the contractile, α-actin isoform. α-Actin is normally expressed by SMC in the vascular wall, but it is slowly lost in cell culture. It can be reinduced by serum-deprivation and TGF-β1 treatment, independent of TGF-β1's effect upon cell cycle. We observed previously that 30–40% of lesion-derived cells spontaneously express some level of α-actin immunoreactivity (4). As shown in Fig. 2, TGF-β1 caused a strong increase in the expression of the 45-kD α-actin protein by Western blot (*inset*). Immunofluorescence indicated that the majority of untreated cells expressed a low level of α-actin in a diffuse cytoplasmic form, but after TGF-β1 treatment the cells showed a marked increase in the level of α-actin, condensation of the α-actin into cytoplasmic fibers, and increased cell spreading. Thus, lesion cells make a prominent phenotypic change in response to TGF-β1, despite their absence of an antiproliferative response to TGF-β1.

Induction of plasminogen activator inhibitor-1 in response to TGF-β1

Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor of tissue-type plasminogen activator and uPA, which converts plasminogen to the active serine protease, plasmin. Thus, induction of PAI-1 activity by TGF-β1 is considered an important aspect of its ability to favor matrix accumulation, by preventing matrix degradation. The ability of TGF-β1 to induce PAI-1 activity was examined in normal and lesion-derived cells. As shown in Fig. 3, PAI-1 activity is significantly induced in both cell types, though the lesion cells show their maximal stimulation at a lower dose of TGF-β1 than normal SMC.

The effect of TGF-β1 on extracellular matrix synthesis in lesion and normal cells

In addition to inhibiting the degradation of extracellular matrix, TGF-β1 induces de novo synthesis of key matrix components such as collagen, fibronectin, thrombospondin, and proteoglycans. [^{3}H]Proline incorporation into insoluble, cell-associated protein was used as a quantitative marker of matrix synthesis because as much as 80% of proline is incorporated into collagen. We have documented previously the synthesis of type I and III collagens by specific RIA and Western blots, as well as the conversion of [^{3}H]proline to [^{3}H]hydroxyproline in lesion-derived cells (4).

As shown in Fig. 4, lesion-derived cells showed a strong induction of [^{3}H]proline incorporation into cell-associated proteins in response to TGF-β1. Two different lesion cell lines exhibited dose-dependent increases in [^{3}H]proline incorporation of 1.8–2.6-fold higher than basal levels at 1 ng/ml of TGF-β1 ($P < 0.01$). In contrast to the increase observed in lesion cells, normal SMC showed a small decrease in [^{3}H]proline incorporation to 70% of control levels during the 72-h treatment period that was not statistically significant.

The incorporation of [^{3}H]proline into soluble proteins in normal cells was not significantly affected at any concentration (1 ng/ml: 95% of control±16 SEM, P = NS) while only cells from lesion 2 showed significant increases, and only at the highest dose of TGF-β1 (1 ng/ml: lesion 2 = 151±7.7%, $P < 0.05$; lesion 3 = 117±1.4%, P = NS). Baseline rates of [^{3}H]proline incorporation into soluble or cellular protein differed significantly only between the normal cells and cells from lesion 2 (cell-associated: normal = 9,680±1,858 vs. lesion 2 = 2,975±202 dpm/well; $P < 0.05$). Baseline incorporation into soluble or cellular protein was not significantly different between normal cells and the lesion 3 cells.

Analysis of membrane receptors for TGF-β1 by radioligand cross-linking

Considering the differences in the functional responses to TGF-β1, the membrane receptor pattern was examined in normal and lesion cells by ^{125}I-TGF-β1 cross-linking and SDS-PAGE. As shown in Fig. 5, normal cells express all three membrane receptors, while the lesion cells show a major reduction in the type

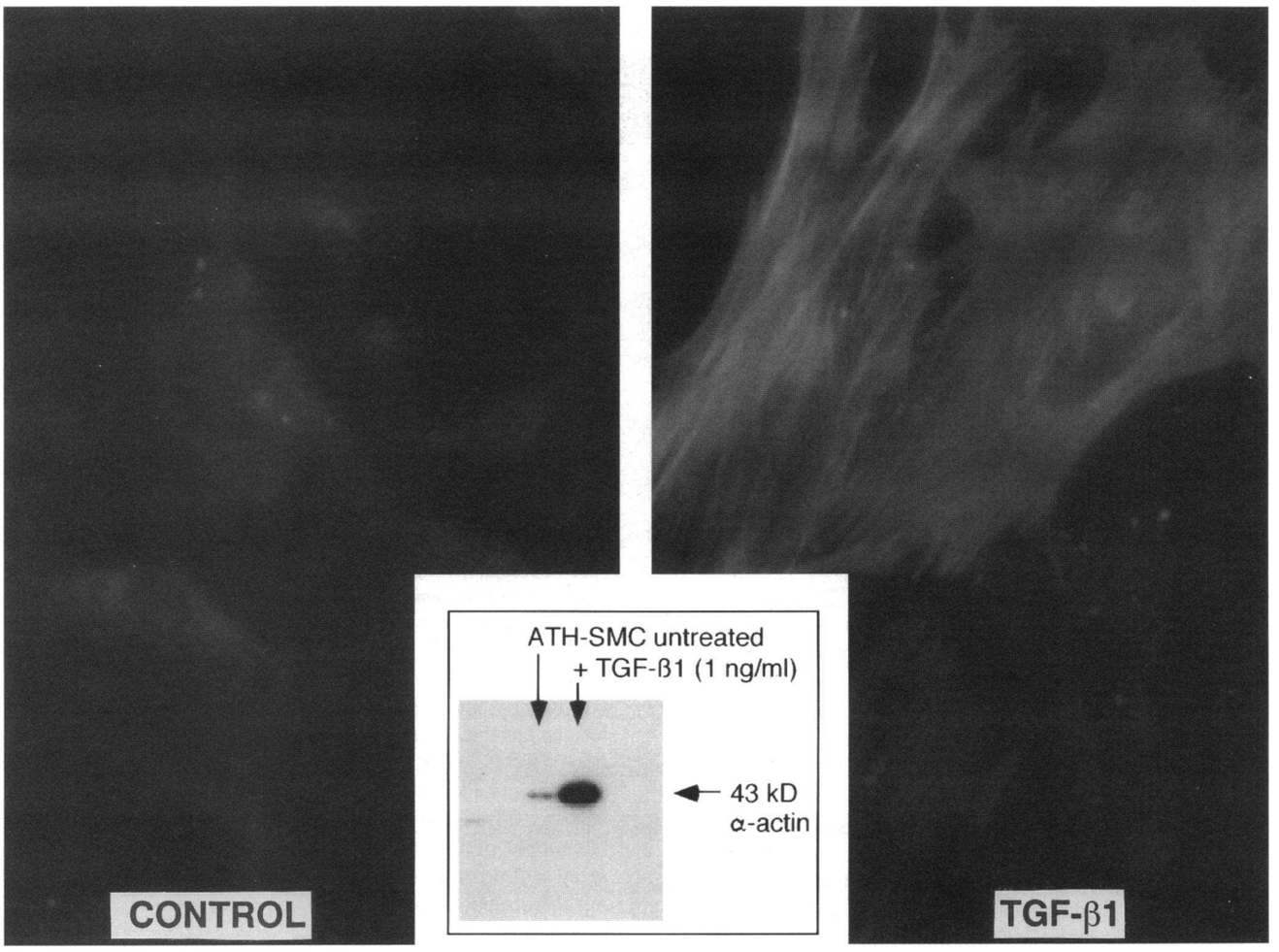

Figure 2. The effect of TGF-β1 on the expression of the contractile α-actin isoform in cells derived from vascular lesions. Cells derived from a superficial femoral artery endarterectomy (patient 3) were plated on chamber slides for 24 h in 10% FBS and then changed to 1% FBS for 72 h without (*CONTROL*) or with 1 ng/ml TGF-β1 (*TGF-β1*). Cells were fixed with 4% buffered formaldehyde and stained with a monoclonal antibody to the contractile α-actin isoform detected by FITC-labeled goat anti-mouse antibody. Nonimmune IgG controls were negative. (*Inset*) Cells grown from a restenotic coronary artery lesion were treated for 24 h before analysis by Western blot for α-actin, detected by ^{125}I-protein A.

II receptor with little or no change in the type I receptors. Quantitative analysis of the cross-linking gel indicated that radioactivity in the band corresponding to the known type II receptor was 320% higher in normal than lesion cells, while binding to the type I receptor was only 22% higher in normal cells. Normal SMC demonstrated a ratio of type II to I receptors of 3.2:1, while lesion cells showed a ratio of only 1.2:1.

Binding to the high molecular weight type III receptor was somewhat higher in normal than lesion cells (171% of binding to lesion cells). Lesion cells, however, showed a fivefold increase in very high molecular weight binding sites, which remained near the gel origin. This increase in very high molecular weight binding sites may be due to differences in the extracellular matrix, but probably does not explain the decrease in binding to the type II receptor, because binding to the type I receptor is relatively unchanged.

Analysis of receptor mRNA by RT-PCR

The expression of mRNAs for the type II and I TGF-β1 receptors, and TGF-β1, was examined by RT-PCR because prior studies (18), and our preliminary studies, indicated that 2 μg of poly $(A)^+$ mRNA is necessary to detect the type II receptor mRNA by Northern blot in SMC, an amount which exceeds the cell numbers produced from lesion or normal SMC cultures. Based on the similarity in production of TGF-β1 activity, primers for the TGF-β1 mRNA were used to confirm that equal amounts of mRNA were present in normal and lesion cells. As shown in Fig. 6, a band of the predicted size (244 bp) from the TGF-β1 mRNA was equally amplified in both cell types. A band ~ 50–60 bp larger than the predicted size was observed in both normal and lesion cells. The 5′ PCR primer anneals in exon 6 and the 3′ primer anneals in exon 7 of the human TGF-β1 gene, spanning a 50-bp intron (26). Thus, this band probably reflects amplification of the unedited, primary mRNA transcript.

The type I receptor mRNA (252 bp) was amplified essentially equally from both lesion and normal cells, while the type II receptor (784 bp) was much more abundant in normal than lesion cells, which agrees with the pattern of cross-linking to the membrane proteins coded by these mRNAs. This difference in the steady state level of the type II receptor mRNA was observed in at least three experiments analyzing different normal and lesion cell lines (both coronary and peripheral arteries), while the type I receptor was consistently equal. Parallel reactions omitting the RT, or using heat-denatured RT, did not

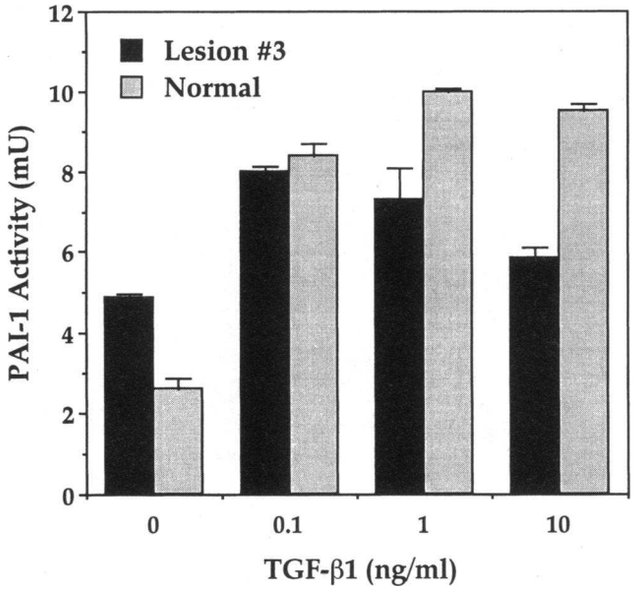

Figure 3. Induction of PAI-1 activity in normal and lesion cells by TGF-β1. Cells derived from a superficial femoral artery endarterectomy (patient 3) and cells from a normal artery were treated with increasing concentrations of TGF-β1 in serum-free media. Supernatants were assayed for PAI-1 activity by their ability to inhibit uPA activity as described in Methods. Points reflect mean±SEM, n = 3 per group.

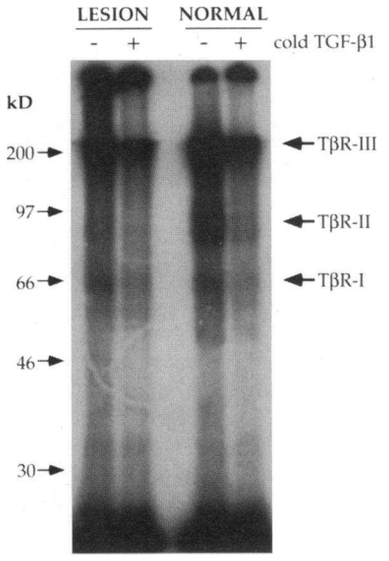

Figure 5. Identification of membrane receptors for ^{125}I-TGF-β1 by radioligand cross-linking. Cells from a femoral artery lesion (patient 3) or normal human aortic SMC were exposed to ^{125}I-TGF-β1 under equilibrium conditions at 4°C for 4 h followed by cross-linking with DSS, Triton X-100 extraction of the membranes, and SDS-PAGE under reducing conditions. The dried gel was exposed to film and analyzed by PhosphoImager. The position of ^{14}C-labeled standards is shown on the left (kD) and the position of the specific receptors is shown on the right. Binding was conducted in the absence (−) or presence (+) of a 50-fold excess of unlabeled TGF-β1 to determine specificity.

amplify bands of the predicted size, indicating that DNA was not amplified in the RT-PCR under these conditions.

The identity of selected type I and II RT-PCR products was confirmed by dideoxy sequencing of portions of subcloned products from normal SMC. Likewise, the identity of both type I and II receptor RT-PCR products was confirmed and semiquantitated by hybridization with [^{35}S]ATP-labeled cDNA probes and counting on a PhosphoImager (Molecular Devices). The type I receptor was found to be essentially equal between lesion and normal cells. In contrast, the type II receptor was consistently much lower in coronary and peripheral vascular lesion cells than in normal aortic SMC. Southern blot hybridization of the RT-PCR products shown in Fig. 6, and other similar experiments, indicated that the type II receptor RT-PCR product was > 10-fold lower in lesion cells than normal cells. The magnitude of this difference was corroborated by analyzing serial 10-fold dilutions of the RNA before RT-PCR and examining the disappearance of the PCR product.

Transfection of type II receptors into lesion cells

The decreased expression of both the type II receptor cross-linking site and the type II mRNA suggested that the relatively selective loss of the antiproliferative response to TGF-β1 might

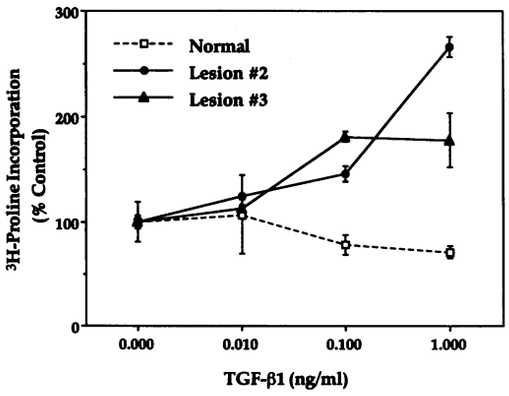

Figure 4. The effect of TGF-β1 on [^{3}H]proline incorporation into cell-associated proteins. Cells derived from two different vascular lesions acquired by endarterectomy (patients 2 and 3) or normal human aortic SMC were treated for 72 h with various levels of TGF-β1 in the presence of [^{3}H]proline. The cells were washed extensively, dissolved in 1 N NaOH, and [^{3}H]proline incorporation was determined by scintillation counting. Points are mean±SEM. dpm per well expressed as a percentage of baseline incorporation (n = 3 per point).

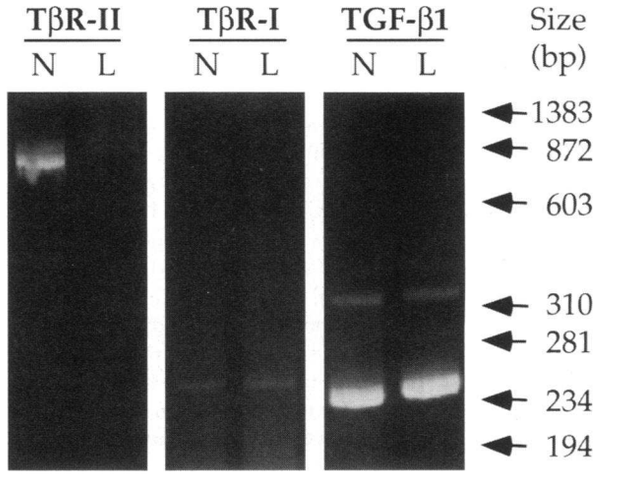

Figure 6. Analysis of TGF-β1 and TGF-β1 receptor mRNAs by RT-PCR. Total RNA was prepared from parallel cultures of normal (N) human aortic SMC and cells derived from a primary atherosclerotic lesion (L) in a human iliac artery. Total RNA was analyzed by RT-PCR for expression of the type II receptor (784 bp), type I receptor (252 bp), and TGF-β1 (244 bp). Positions of size markers are shown on the right (bp).

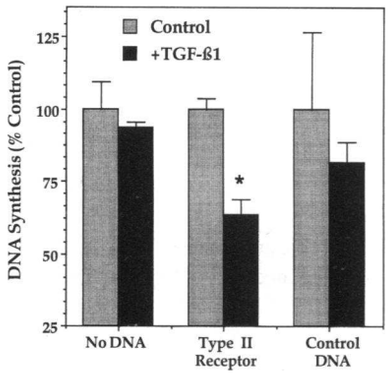

Figure 7. The effect of type II receptor transfection on the antiproliferative response to TGF-β1 in lesion-derived cells. Cells derived from a de novo coronary artery lesion acquired by directional atherectomy were transfected with a plasmid (pcDNA 1) bearing the full-length human type II receptor cDNA. Control groups were either untreated (*No DNA*) or transfected with irrelevant DNA (sheared genomic DNA from 3T3 fibroblasts) in LipofectAmine for 24 h, followed by a 24-h recovery period in serum-containing media. Cells were then treated with TGF-β1 (1 ng/ml) for 18 h followed by a 4-h pulse of [^{3}H]thymidine. Bars reflect cpm/well expressed as a percentage of control (mean±SEM, n = 3). Asterisk indicates that TGF-β1 caused a significant inhibition in type II receptor transfectants ($P < 0.05$).

be linked to the loss of type II receptor, as suggested by other studies (27). To further test this idea, cells derived from a de novo coronary artery lesion were transfected with a mammalian expression vector bearing the human type II receptor cDNA (clone H2-3FF) which encodes the 92.5-kD cross-linked band on SDS-PAGE (18). As shown in Fig. 7, transfection of the type II receptor was associated with a significant antiproliferative response to TGF-β1 (37% inhibition, $P < 0.005$). Similar results were observed by transfection of the type II receptor into cells derived from a restenotic human coronary lesion. Transfection of control DNA under the same conditions was not associated with a significant antiproliferative response to TGF-β1 (18% inhibition, $P > 0.25$). Baseline DNA synthesis was also affected by type II receptor transfection (no DNA = 271±25 cpm/well vs. type II = 141±5 cpm/well), though the transfection of irrelevant DNA also caused a decrease in DNA synthesis (171±45 cpm/well).

The efficiency of lipid-mediated transfection in these cells was evaluated with a similar plasmid vector, pcDNA3, bearing the lacZ reporter construct. Under similar transfection conditions, ~ 30% of cells were lacZ positive, which is consistent with the antiproliferative response induced in the transfected cells (37% inhibition in transfectants versus 7% inhibition in nontransfectants). This suggests that after the introduction of a functional type II receptor, lesion-derived cells are at least partially capable of transducing an antiproliferative signal.

Discussion

The response to TGF-β1 by cells within vascular lesions would be one important aspect of the wound healing response that could govern the progression of both atherosclerosis and restenosis. The existence of a subtype of cells with an aberrant fibrotic and proliferative response to TGF-β1 could explain the slow accumulation of extracellular matrix during these processes. The present results document the existence of such a cell type proliferating from vascular lesions and the association of their dysfunctional responses with an altered pattern of TGF-β1 receptors. The importance of TGF-β1 in the formation of vascular lesions is supported, in part, by evidence that when infused into rats with preexisting vascular lesions, TGF-β1 causes an 80% increase in lesion size due primarily to extracellular matrix accumulation (9). Likewise, transfection of the cDNA for TGF-β1 at the time of balloon injury to porcine arteries causes marked increases in the matrix content of the resulting lesion (14).

Prior studies indicated that cells grown from human restenotic vascular lesions were unresponsive to the antiproliferative effects of heparin, which inhibits the growth of SMC from normal arteries (17). This result is consistent with the present data indicating that cells from vascular lesions are resistant to inhibition by TGF-β1 and is also consistent with evidence that there is a relationship between the antiproliferative effects of heparin and TGF-β1 (28–31). The resistance to these two inhibitors may be explained by changes in the TGF-β1 receptors or by changes in intracellular signaling systems that impact on their respective inhibitory pathways. Together, these results define important alterations in the response of cells in lesions to growth inhibitors and suggest a possible relationship of inhibitor resistance to the pathobiology of vascular disease.

Coincident with the loss of antiproliferative response to TGF-β1, the cells grown from lesions show a gain of response to TGF-β1 for synthesis of extracellular matrix proteins. The profibrotic response to TGF-β1 is due to two main effects: induction of new matrix synthesis and suppression of matrix degradation. In the latter case, TGF-β1 is a natural stimulus for PAI-1, acting via specific TGF-β1 responsive elements in the 5′ promoter region of the PAI-1 gene (32). The induction of PAI-1 suppresses plasminogen conversion to plasmin, which has the effect of (*a*) reducing degradation of the extracellular matrix; (*b*) suppressing the activation of collagenases from procollagenases (33); (*c*) inhibiting autocrine/paracrine activation of latent TGF-β (34); and (*d*) blocking plasmin-mediated release of matrix-bound growth factors (35). Matrix degradation by collagenases is further inhibited by tissue inhibitor of metalloproteases, which is potently induced by TGF-β1 (36).

The second major profibrotic effect in fibroblasts, in particular, is the induction of the matrix components: fibronectin, collagen, elastin, thrombospondin, and proteoglycans (37, 38). The induction of collagen synthesis is probably regulated independently of PAI-1 because type I collagen gene regulation by TGF-β1 appears to be mediated by nuclear factor-1 promoters (37), while the effect of TGF-β1 on the PAI-1 gene is thought to be mediated via AP-1 sequences (39). In normal rabbit SMC, TGF-β1 induces a fourfold increase in thrombospondin mRNA, a twofold increase in α1(IV) collagen mRNA levels, but has very minor effects on other matrix constituents such as α2(I), α1(III), and α2(V) collagen mRNAs or fibronectin mRNA (40). Based on the results in fibroblasts, as well as the effects of TGF-β1 on the vascular lesion in vivo, it is surprising that TGF-β1 has little effect on [^{3}H]proline incorporation in normal SMC. However, the lesion-derived cells showed a prominent profibrotic response to TGF-β1, showing 1.8–2.8-fold increases in [^{3}H]proline incorporation into cell-associated protein at doses of 1 ng/ml or less. Combined, these data indicate that the fibrotic effects of TGF-β1 in vascular plaques are due potentially to both PAI-1–mediated inhibition of matrix degradation and de novo matrix synthesis in response to TGF-β1.

The current data provide further evidence that the antiproliferative and fibrotic effects of TGF-β1 can be modulated independently (41), either by alterations in the signaling pathways (42) or by selective changes in the type II/type I receptor balance (27). It is known that normal SMC modulate their

response to TGF-β1 and their membrane receptors with increasing cell density in vitro (43). Both of the low molecular weight receptors are serine-threonine kinases that appear to signal through heteromeric complexes. The current model of type II/type I signaling predicts that the type II receptor is required for TGF-β1 presentation to the type I receptor (44). The type II receptor has been most closely associated with changes in cell proliferation, and the type I receptor appears necessary for the induction of protein synthesis. This is based, in part, on transfection of dominant negative receptors, which indicated that the effect on DNA synthesis can be separated from the effects on the induction of PAI-1 (27). The present studies largely support this model, as the down-regulation of the type II receptor in lesion cells is associated with loss of an antiproliferative response to TGF-β1. Conversely, the type I receptor cross-linking site is retained in lesion cells, the type I mRNA appears unchanged between cell types, and the cells show similar induction of PAI-1 activity.

Two findings, however, are not clearly explained by the existing model. First, why does down-regulation of the type II receptor, which is thought to act as a prerequisite for type I receptor binding, have so little effect on type I binding? A plausible answer is that, while markedly decreased, the type II receptor remains at a level which is not rate-limiting for type I binding, but is insufficient for adequate type II responses to be transmitted. Secondly, why do two different cells lines, lesion and normal cells, with similar levels of the type I binding site show such different type I–mediated effects on extracellular protein synthesis? It is interesting to speculate that the type II or III receptors influence the type I–mediated response, or that the induction of extracellular matrix synthesis requires additional transcriptional activators to facilitate TGF-β1 stimulation.

Several different models may explain the resistance of the lesion-derived cells to TGF-β1. First, these refractory cells may reflect a subset of SMC derived from the underlying normal artery that have been selected over time by their resistance to inhibition. Two lines of evidence support this "selection by aging" model. First, in animal models, where anatomical origin, genetic background, and pathogen exposure are identical, older animals show an excessive response to vascular injury (45, 46), and SMC derived from old Fisher 344 rats ($>$ 20 mo) are growth stimulated by TGF-β1 while cells from young adult rats ($<$ 3 mo) are growth inhibited (23). Second, SMC in culture typically lose responsiveness to TGF-β1 with repeated subpassage (McCaffrey, T., unpublished observation). Likewise, subculture of human colon carcinoma cells leads to spontaneous subclones that are resistant to the antiproliferative effect of TGF-β1 (47). Thus, selection may occur for cells with low type II receptor expression, or alternatively, selection may occur for some related function which is coupled to type II receptor expression.

In a second model, these refractory lesion-derived cells may represent a fundamentally different cell type, derived from tissue or the circulation, such as a myofibroblast, which might not normally exhibit an antiproliferative response to TGF-β1. A third model is suggested by the fact that certain pathogens have the ability to alter the cellular response to TGF-β1. Resistance to TGF-β1 can be imparted by the adenovirus *E1A* oncogene (48) and by HPV-16, HPV-18, and SV40, which produce proteins that interact with the retinoblastoma gene product (49). Likewise, the IE84 protein product of cytomegalovirus, which has been detected in a subset of restenotic lesions, can bind and neutralize p53 (50). Inactivation of p53 is associated with refractoriness to TGF-β1 (51). Thus, it is interesting to speculate that a viral factor may be able to confer resistance to the antiproliferative effect of TGF-β.

A fourth possibility is that the type II receptor gene is inactivated due to an acquired mutation. This is supported by recent data showing that a class of colon carcinoma cell lines exhibits point mutations in two highly repeated domains in the coding region of the type II receptor gene (52). This microsatellite instability appears to result from a deficiency in the correction of replication errors and is associated with the absence of a growth inhibitory response to TGF-β1 and loss of the type II receptor and its mRNA. These various models are not mutually exclusive and, thus, it is interesting to speculate that the restenotic lesion may result from a small number of cells with a mutational inactivation of their type II receptors that are amplified due to their resistance to inhibition.

Regardless of the precise mechanistic origin for the functional and receptor differences, these changes have important implications for the disease process and the way it is studied. The behavior of these lesion cells in response to TGF-β1 is consistent with the pathogenesis of atherosclerosis and restenosis: slow, unregulated growth coupled with excessive extracellular synthesis and inhibition of matrix degradation. The fact that the response of the lesion cells to TGF-β1 is essentially the reverse of normal SMC suggests that caution should be applied when extrapolating from the study of normal human or animal SMC to the process of atherosclerosis or postangioplasty restenosis. A more extensive examination of lesion-derived cells may allow insights into differences that might be exploited in the control of fibrovascular diseases.

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