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Evidence for an Age-Related Dysfunction in the Antiproliferative Response to Transforming Growth Factor-β in Vascular Smooth Muscle Cells

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Previous studies have indicated that aged animals show an increased intimal hyperplasia after arterial injury. The present studies examined the hypothesis that the increased serum-free proliferation of aged smooth muscle cells (SMC), in vitro, was due to a loss of an antiproliferative signal, such as transforming growth factor-β1 (TGF-β1). Northern blot analysis of the mRNA derived from old (>19 mo) or young (3–4 mo) rat aortic SMC indicated that both groups had an equivalent level of the 2.5 kB TGF-β1 message. Metabolic labeling with ^{35}S-methionine and immunoprecipitation for TGF-β1 confirmed the de novo synthesis of TGF-β1 in rat SMC. Old and young SMC supernatants showed equal levels of active or latent (acid-activated) TGF-β activity. Despite the similarities in the production of TGF-β1, old SMC were refractory to inhibition by TGF-β1, whereas young SMC were markedly inhibited (80%) by low levels of TGF-β1 (IC_{50} < 5 pg/ml). Binding studies at 4°C indicated that old SMC exhibited reduced binding capacity for ^{125}I-TGF-β1. Cross-linking studies confirmed that old SMC showed reduced binding of ^{125}I-TGF-β1 to membrane sites corresponding to the high molecular weight type III receptor, as well as the 85-kDa type II and 65-kDa type I receptor. However, at 37°C, old SMC degraded ^{125}I-TGF-β1 more rapidly than young SMC. Combined, this data suggests that SMC derived from older animals are capable of normal production of TGF-β1 but fail to respond to the autocrine growth inhibitory effects of this agent, thereby leading to enhanced proliferation.

INTRODUCTION

Advancing age is a strong risk factor for atherosclerosis. By the age of 65 years, as much as 50% of the coronary artery surface can be covered by raised lesions (World Health Organization, 1976). Accumulating evidence indicates that the vascular smooth muscle cell (SMC) is an early component of the atherosclerotic plaque in humans (Gown, 1992). Likewise, within 6 months after balloon angioplasty, 35% of patients develop a proliferative restenotic lesion that is composed largely of SMC (Dartsch *et al.*, 1990). Thus, the influence of age on SMC proliferation is potentially important both to the pathogenesis of atherosclerosis and to its successful treatment.

Increasing evidence indicates that age-related atherosclerosis may be due to intrinsic changes in the arterial wall that exaggerate the response to vascular injuries, including hypercholesterolemia (Spagnoli *et al.*, 1991). After balloon catheter injury to the aorta, aged rats show an excessive and prolonged intimal hyperplasia compared with younger controls (Stemerman *et al.*, 1982; Hariri *et al.*, 1986). Transplant studies indicate that this excessive intimal response is the result of the age of the artery itself and not due to variations in systemic factors (Hariri *et al.*, 1986). Our laboratory and others have observed that this phenomenon could be attributable to the increased proliferation of isolated SMC derived from aged rats (>20 mo) (Hariri *et al.*, 1988; McCaffrey *et al.*, 1988). Under low-serum conditions, in which SMC proliferation is governed by the balance of autocrine mitogenic and inhibitory signals, SMC derived from aged rats proliferate at a 2.4-fold higher rate than young SMC (McCaffrey *et al.*, 1988). It is known that these cells are producing platelet-derived growth factor (PDGF)-like mitogens that would contribute to their serum-free proliferation (McCaffrey *et al.*, 1988; Majack *et al.*, 1990) and that this age-related increase in prolif-

erative rate is corrected by heparin (McCaffrey *et al.*, 1988).

We have observed recently that transforming growth factor-β1 (TGF-β1) is a strong heparin-binding protein (McCaffrey *et al.*, 1992b) that appears to partially mediate the antiproliferative effect of heparin in vascular SMC (McCaffrey *et al.*, 1989). TGF-β1 is a potent modulator of cell proliferation, extracellular matrix accumulation, and chemotaxis (Sporn *et al.*, 1987; Wakefield *et al.*, 1988) that is overexpressed in human coronary restenotic lesions (Nikol *et al.*, 1992). The present studies assess whether differences in the production of, or response to, TGF-β1 is a factor in the accelerated proliferation of aged SMC. The data indicate that although TGF-β1 is produced equally by both old and young SMC, the binding, processing, and biological response to TGF-β1 differ markedly. Because TGF-β1 is an autocrine inhibitor of SMC proliferation in vitro (McCaffrey *et al.*, 1989), we postulate that escape from TGF-β's antiproliferative effect by negative selection of sensitive cells is a possible explanation for the dysregulated growth of SMC from aged animals.

METHODS

Reagents

TGF-β1, derived from porcine platelets, and antibodies (rabbit and chicken anti-porcine TGF-β1) were purchased from R&D Systems (Minneapolis, MN), and for selected experiments human recombinant TGF-β1 was kindly provided by Berlex Biosciences (S. San Francisco, CA). ^{3}H-Thymidine (20 Ci/mmol), ^{125}I-Na, ^{3}H-glucosamine (40.0 Ci/mmol), ^{3}H-proline (25–55 Ci/mmol), and ^{35}S-methionine (>800 Ci/mmol) were purchased from DuPont New England Nuclear (Wilmington, DE). Bis(sulfosuccinimidyl) suberate (BS^3), a bifunctional cross-linking agent, was purchased from Pierce Chemicals (Rockford, IL).

Cell Culture

Rat aortic SMC were subcultured from explants of the dissected medial portion of the thoracic aortas from young (<3 mo) or old (>19 mo) Fisher 344 male rats (Harlan Sprague Dawley, Indianapolis, IN). In early passage (<p3) these cells express SMC-actin as determined by immunoreactivity with smooth muscle-specific actin antibody (Sigma Chemicals, St. Louis, MO). SMC were cultured in medium 199 with 10% fetal bovine serum (FBS), gentamicin sulfate (50 μg/ml) and amphotericin B (Fungizone, 2.5 μg/ml). Subculture was achieved with trypsin/EDTA and a 3:1 split ratio.

Northern Blot Analysis

RNA from SMC was prepared by the guanididium isothiocyanate/cesium chloride method. Equal numbers of cells were harvested (1–2 × 10^6 cells/75-cm^2 flask) by washing with *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered saline (HBS) and dissolving directly with 2–4 ml of 4 M guanididium isothiocyanate. After an 18-h centrifugation through a CsCl gradient, the RNA was quantified by absorbance at 260 and 280 nm. Between 10 and 30 μg of total RNA per lane was electrophoresed in a 1% agarose gel and then transferred to nitrocellulose. Equal loading of lanes was determined by densitometric analysis of photographs of the gel under ultraviolet (UV) light and, in some cases, confirmed by probing the blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The blot was hybridized with a TGF-β1 RNA probe (Derynck *et al.*, 1986) prepared by ^{32}P-UTP labeling from a DNA template bearing the SP6 promoter (kindly provided by R. Derynck, Genentech, San Francisco, CA). Relative levels of TGF-β1, GAPDH, and 28S mRNA (by UV) were assessed by densitometric scanning using a UMAX (Santa Clara, CA) UC630 flatbed scanner attached to a Macintosh IIci (Apple Computer, Cupertino, CA) running NIH Image analysis software (Masters *et al.*, 1992).

Metabolic Labeling/Immunoprecipitation

The de novo synthesis of TGF-β1 was examined by metabolically labeling SMC (2 × 10^6 cells/75-cm^2 flask) with 25 μCi/ml of ^{35}S-methionine in methionine-free Eagle's minimum essential medium for 64 h. The cells were quickly washed and then scraped into 3 ml of phosphate-buffered saline (PBS) with 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% Tween-80, and 0.1% Triton X-100 (PTT). The cells were then sonicated for 2 min on ice, acidified to pH 3.0 for 20 min with 150 mM HCl, and then neutralized with NaOH to pH 7.0. An aliquot of the lysate (400 μl) was precipitated with either 75 μl of control buffer (medium 199 = no antibody), 50 μl of anti-TGF-β1 (1 mg/ml stock of a neutralizing antibody raised in chickens; R&D Systems), or the antibody with 25 μl of 1 ng/μl of TGF-β1 (blocked). The antibody was then precipitated with 50 μl of 50% Protein A-agarose slurry overnight at room temperature. Lysates from unlabeled SMC were used to block nonspecific sites on the Protein A beads. The Protein A agarose was then washed five times with 750 μl of PBS + PTT, and the labeled proteins were eluted with nonreducing sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then electrophoresed through an 8–18% polyacrylamide gel. The gel was treated with a precipitable scintillant (Enhance, Dupont New England Nuclear, Wilmington, DE), washed, and the dried gel was exposed to Kodak X-Omat film (Rochester, NY) with Cronex Lightning Plus enhancing screens (Dupont) at −70°C for 3 wk. The molecular mass of the immunoprecipitated bands was determined by prestained molecular mass markers (Bethesda Research Laboratories, Gaithersburg, MD) and by a parallel lane containing ^{125}I-TGF-β1.

CCL64 Bioassay for TGF-β1 in SMC Supernatants

Serum-free supernatants of old and young SMC (2 × 10^6 cells/75 cm^2) were collected for sequential 48-h periods after a 24-h washout period. Three such 48-h periods were collected from each of four old and three young cell lines of similar passage and frozen at −40°C until assay. Supernatants were centrifuged (10 000 × *g* for 10 min) to remove any cellular debris, and, when appropriate, aliquots were acidified to pH 2.0 with sterile 0.5 M HCl for 1 h and neutralized with sterile 0.5 M NaOH. Supernatants were diluted as necessary (typically 1:10) with CCL64 growth media (medium 199 + 10% FBS + 50 μg/ml gentamicin) and assayed for TGF-β1 activity as previously described (McCaffrey *et al.*, 1992b). The inhibition of CCL64 DNA synthesis is extremely sensitive to both TGF-β1 and TGF-β2 (IC_{50} = 0.05 ng/ml) but is essentially insensitive to other known mitogens or inhibitors (Danielpour *et al.*, 1989). TGF-β1 activity in cell supernatants is computed from a four-parameter fit of a standard curve of TGF-β1 (0.005–2 ng/ml) and corrected for the dilution. Control studies using supernatants with added TGF-β1 failed to detect the presence of any agents (i.e., proteases, binding proteins, or mitogens) that would interfere with the accurate determination of TGF-β1 levels by this method.

Cell Proliferation

The growth of old and young SMC under low serum conditions (Figure 1) was examined by plating the cells at 20 000 cells/well of a 96-well plate in growth media (medium 199 + 10% FBS + antibiotics). After 24 h the cells were changed to medium 199 + 2% FBS + antibiotics + insulin, transferrin, selenium (ITS) (Sigma Chemicals, St. Louis, MO), and cell counts were taken at specified intervals by trypsinization

and physical cell counting in a Coulter ZBI (Coulter Electronics, Hialeah, FL) cell counter. The response to TGF-β1 (see Figure 4) was determined by plating old and young SMC at a density of 1×10^4 cells/well of 96-well plate in growth media. After a 48-h plating period, the cells were exposed to increasing concentrations of TGF-β1 in growth medium for 18 h before a 4-h pulse of ^{3}H-thymidine (0.1 μCi/well). The cells were then washed, trypsinized, and the DNA collected onto glass filtermats with a cell harvester. Thymidine incorporation into DNA was determined in a Wallac RackBeta scintillation counter (Wallac, Gaithersburg, MD).

Binding and Uptake Studies

The interaction of ^{125}I-TGF-β1 with cell surface receptors was examined by plating old and young SMC at 1×10^5 cells/well (2 cm^2) of a 24-well plate in growth medium. Before binding, cell counts were taken in adjacent wells by trypsinization and particle counting (Coulter ZBI), and the remaining cells were changed to serum-free media for ≥30 min. The cells were then moved to a 4°C cold room and equilibrated, and increasing amounts of ^{125}I-TGF-β1 (5850 Ci/mmol) were applied in binding media (medium 199 + 0.1% BSA) for 3 h, at which time the cells were washed five times with cold binding media. The cells were then extracted with a solubilization buffer that minimizes the elution of nonspecific binding (25 mM HEPES, 1% Triton S-100, 10% glycerol) for 30 min at 37°C. Nonspecific binding was assessed by the addition of a 100-fold excess of cold ligand.

Uptake studies were performed to determine the rate of cellular degradation and steady-state levels of cell-associated ^{125}I-TGF-β1 at 37°C. Cells were plated similarly to binding studies, and ^{125}I-TGF-β1 (50 000 cpm/200 μl/well) was added to wells of the 24-well plate in binding media and incubated at 37°C for the specified times. At each time point, the supernatant was removed from duplicate wells, the cells were quickly washed with HBS + 0.1% BSA, which was added back to the supernatant. The cells were washed twice more, and the monolayer was dissolved with 1 N NaOH to determine cell-associated radioactivity. The supernatant was precipitated with an equal volume of 20% trichloroacetic acid (TCA) and then centrifuged (10 000 rpm for 10 min) to determine acid-soluble degradation products (supernatant) and acid-precipitable protein (pellet). Background degradation in the absence of cells due to free iodine or proteolysis during storage was subtracted to yield cell-specific degradation.

The binding of radiolabeled TGF-β1 to cell surface receptors was further characterized by covalently cross-linking the ligand to membrane acceptors. SMC were plated under reduced serum conditions (5% FBS) at 1×10^6 cells/25-cm^2 flask 48 h before use. Binding conditions were similar to the 4°C binding studies described above, except that after the 3-h binding period the cells were washed and treated with 250 μM BS^3 in PBS for 15 min followed by washing with 10 mM tris(hydroxymethyl)aminomethane + 0.1 mM PMSF + 1 mM EDTA, scraping the cells into the same buffer, and extracting with this buffer + 1% Triton X-100 for 30 min at 37°C. The extracted counts were mixed with SDS sample buffer and electrophoresed in a 7% gel with a 4% stacking gel under nonreducing conditions. The gel was dried and exposed to Kodak X-Omat film with enhancing screens for 3 d. Control experiments determined the specificity of the binding by omission of the cross-linker and by cold competition.

Determination of Glycoprotein and Protein Synthesis by Metabolic Labeling

Glycoprotein and protein synthesis were examined by determining the incorporation of ^{3}H-glucosamine and ^{3}H-proline, respectively, into secreted and cell-associated protein. Old and young SMC, plated 48 h previously on six-well plates, were treated with ^{3}H-glucosamine or ^{3}H-proline (1 μCi/ml/well) in serum-free medium 199 with antibiotics for 24 h. Incorporation into soluble proteins was determined by removing the media and precipitating 500 μl of the media with 200 μl of 50% TCA in the presence of 300 μl of 0.2% BSA as carrier. The precipitate was pelleted, washed, and counted in a liquid scintillation counter. Incorporation into insoluble cell-associated protein was determined by washing the cell monolayer once with PBS + 1% BSA and three additional times with PBS alone before dissolving the monolayer with 1.0 N NaOH, neutralizing with 1:1 1 N HCl, and scintillation counting. Incorporation was expressed as DPM per 10^5 cells (n = 4 per group) determined by cell counts in parallel wells.

RESULTS

Differential Low-Serum Growth of Old and Young SMC

The proliferative advantage of old SMC that is observed after balloon injury to the rat aorta is maintained by SMC in cell culture (McCaffrey *et al.*, 1988). Previously, SMC cultured under serum-free conditions showed an initial period of cell loss by both old and young SMC followed by an increased rate of proliferation of the old SMC (McCaffrey *et al.*, 1988). Those results are confirmed and extended in the present studies where it is observed that under low-serum conditions the initial cell loss is avoided and the excessive proliferative rate of the old SMC is maintained. After an initial plating at 2.0×10^4 cells/well, old SMC cultured in 2% FBS/ITS showed a significantly higher cell count at 4, 10, and 17 d postplating than did matched young SMC ($p < 0.005$) (Figure 1). Earlier studies suggested that this type of proliferation might be due to the presence of PDGF-like molecules in the aged SMC cultures, but could not exclude a deficiency in an autocrine inhibitor of SMC proliferation, such as TGF-β1. Thus, similar cell lines were examined for their production of TGF-β1.

Production of TGF-β1 by Rat SMC

TGF-β1 activity in the cellular environment is influenced by steady-state levels of the mRNA, posttranslational

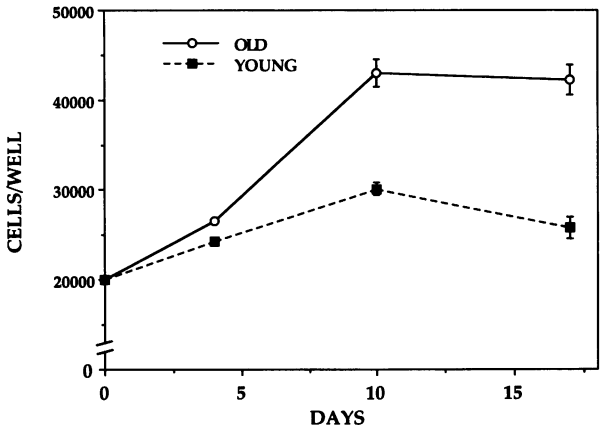

Figure 1. The effect of age on the low-serum proliferation of vascular smooth muscle cells. The proliferation of SMC isolated from old (>19 mo) or young (<4 mo) male Fisher 344 rats was examined by plating at 20 000 cells/well of a 96-well plate on day 0 in medium 199 + 2% FBS + ITS. Cell counts were determined at specified intervals by trypsinization and cell counting in a Coulter ZBI particle counter. Data reflect the mean ± SE (n = 8). Error bars at 4 d are similar to the size of the point marker.

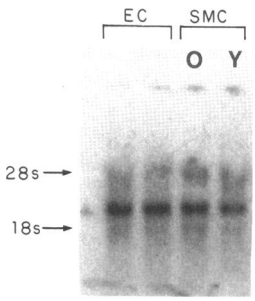

Figure 2. Northern blot analysis of TGF-β1 mRNA in old and young SMC. Human umbilical vein endothelial cells (EC, left lanes) and aortic SMC derived from old or young rats (right lanes) were dissolved in 4 M guanididium isothiocyanate and RNA prepared by cesium chloride gradient centrifugation. Blots were probed with an antisense RNA probe to the 2.5-kb TGF-β1 message, and equal RNA loads were confirmed by densitometric analysis of GAPDH hybridization or by UV fluorescence of ethidium bromide-stained gels.

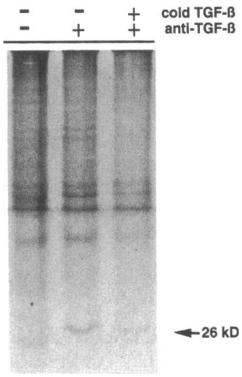

Figure 3. Immunoprecipitation of metabolically labeled TGF-β1 from rat aortic SMC. SMC (young) were metabolically labeled with ^{35}S-methionine (25 μCi/ml) for 64 h before precipitation with an antibody to TGF-β1 with (+) or without (−) an excess of cold TGF-β1 (25 ng). Precipitated proteins were electrophoresed through an 8–18% SDS-polyacrylamide gel under nonreducing conditions and exposed to film for 3 wk at −70°C. Molecular mass was determined by prestained markers and by ^{125}I-TGF-β1 in parallel lanes.

processing, and by conversion of the latent pro-TGF-β1 complex to its active 26-kDa form (Sporn *et al.*, 1987). The TGF-β1 mRNA is a 2.5 kilobase (kb) message (Derynck *et al.*, 1986) that is expressed in a variety of cell types. The regulation of the TGF-β1 mRNA, subsequent protein synthesis, and TGF-β1 activity in SMC cultures was evaluated with respect to aging.

Northern Blot Analysis. In a series of experiments examining SMC derived from aged or young rats, it was observed that SMC express the 2.5-kb TGF-β1 mRNA at levels comparable with that of human umbilical vein endothelial cells as shown in Figure 2. No differences in the steady-state levels of TGF-β1 mRNA between old and young SMC could be detected (<5% difference by densitometric analysis of 3 separate experiments), regardless of whether ethidium bromide staining or GAPDH levels were used to correct for minor loading differences.

Metabolic Labeling/Immunoprecipitation. The absence of differences in steady-state mRNA levels does not preclude differential rates of synthesis, and, in fact, the synthesis of TGF-β1 by rat SMC has not been established previously. Thus, the de novo synthesis of TGF-β1 by rat SMC was examined by metabolic labeling with ^{35}S-methionine followed by immunoprecipitation of the cellular material with an antibody to TGF-β. The results indicate that rat aortic SMC produce a 26-kDa labeled species that is specifically immunoreactive with a neutralizing antibody to TGF-β1 (Figure 3). This band was absent without antibody and was blocked when the antibody was blocked with an excess of unlabeled TGF-β1. A fainter band was immunoprecipitated from old SMC by both chicken antibodies and by rabbit anti-TGF-β. Because these antibodies have relatively low affinity for rat TGF-β and because the levels of TGF-β in SMC conditioned media are quite low (<0.5 ng/ml), we determined TGF-β activity produced by the cells in a more sensitive assay system.

Bioassay of Serum-Free Media and Cell Lysates. The production of TGF-β1 by old and young SMC was examined quantitatively by bioassay of serum-free conditioned media (Table 1). Both old and young showed significant levels of TGF-β1 activity in the cell supernatants (186 and 174 pg/ml, respectively), although no difference between the age groups could be discerned ($p > 0.05$). Upon acid-activation of the supernatant, more than threefold higher levels were observed, consistent with the fact that the majority of TGF-β1 is produced in a biologically latent form due to binding of a precursor sequence (Miyazono *et al.*, 1990). The TGF-β1 activity in old and young supernatants was completely reversed by prior treatment with a neutralizing rabbit antibody (R&D Systems) showing specificity for both the β1 and β2 isoforms of TGF-β.

The Response to TGF-β1 in Old and Young Rat SMC

The Effect of In Vivo Aging on the Response to TGF-β1. Although the production and activation of TGF-β1 does not seem to explain the differential proliferation of old and young SMC, a second alternative is that these cells respond differently to TGF-β1. This was tested directly by determining the effect of increasing doses of TGF-β1 on the rate of DNA synthesis. In young SMC, TGF-β1 was a potent antiproliferative factor with an apparent half-maximal inhibition observed at <5 pg/ml (Figure 4). In contrast, SMC derived from old animals

Table 1. The effect of in vivo age on the production of TGF-β1 by vascular SMC

	SMC		
	Old	Young	p
Active TGF-β1 (ng/ml)	0.186 ± 0.040	0.174 ± 0.057	NS
Total TGF-β1 (ng/ml)	0.622 ± 0.084	0.674 ± 0.096	NS

The levels of TGF-β1 in serum-free supernatants of SMC derived from old (>19 mo) and young (3–4 mo) rats was examined in CCL64 inhibition assay. Total TGF-β1 was measured in the same supernatants activated by acidification to pH 2.0 for 1h before neutralization. Values are mean ± SE of three serial supernatant collections (48 h) on four old and three young cell lines of similar subpassage. Between-group differences were not statistically significant (NS) if $p > 0.05$, $df = 28$.

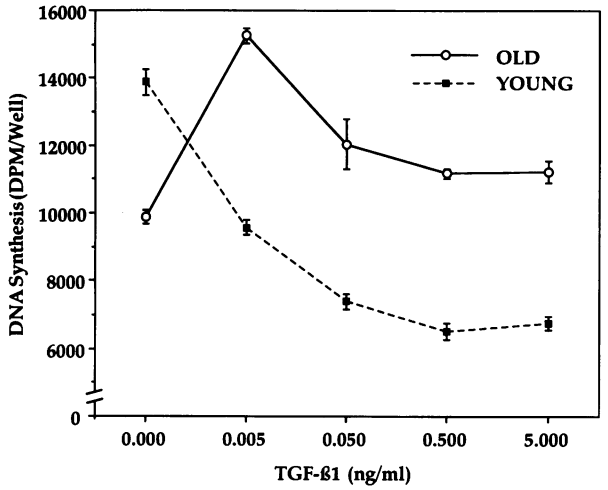

Figure 4. The effect of in vivo age on the cellular response to TGF-β1. SMC derived from old or young SMC were plated at 1×10^4 cells/well of a 96-well plate in medium 199 + 10% FBS. After 48 h they were exposed to increasing concentration of TGF-β1 in the same media for 18 h before a 4-h pulse of ^{3}H-thymidine followed by collection of the labeled DNA and scintillation counting.

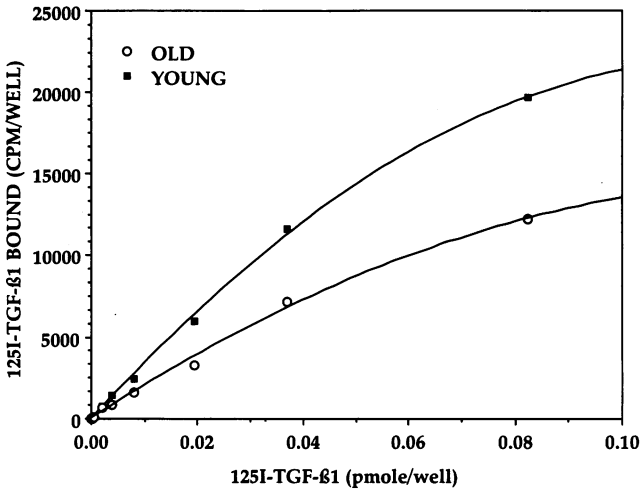

Figure 5. Age-related differences in the binding of ^{125}I-TGF-β1. Equal numbers (1×10^5 cells/24 well) of old and young SMC were plated in growth media and changed to serum-free media 30 min before binding while the cells were equilibrated in a 4°C cold room. Precooled ^{125}I-TGF-β1 (5850 Ci/mmol; 0.08 pmol/well ≅ 11 ng/ml) was added for 3 h before washing and release of bound counts for gamma counting. Points are the average of duplicate wells representative of four separate studies.

were not inhibited by TGF-β1 and were, in fact, modestly stimulated by low concentrations of TGF-β1. Thus, even at extremely low concentrations of TGF-β1, the old SMC showed a significantly higher rate of DNA synthesis and a distinct refractoriness to growth inhibition by TGF-β1. Thus, the increased rate of proliferation by old SMC under low-serum conditions (Figure 1) could be attributed to an absence of an inhibitory response to the 170–180 pg/ml of active TGF-β1 present in such supernatants (Table 1).

The Effect of Age on the Binding of TGF-β. One possible explanation for the differential response to TGF-β1 would be a loss of TGF-β1 receptors. This was examined by radioligand analysis of the cell surface receptors at 4°C. The results of four of five binding studies indicated that within the dose range that produces biological response (0–10 ng/ml), old SMC bound markedly less ^{125}I-TGF-β1 than young SMC of the same subpassage. A representative binding isotherm on equal cell numbers (old, 139 960 cells/well; young, 132 510 cell/2.0-cm^2 well) is shown in Figure 5. Within the biologically active range of TGF-β, saturation binding conditions were not reached, although conditions for equilibrium binding were met, and subsequent Scatchard analysis indicates that both young and old SMC express receptors with K_d of 2.1–4.3 nM, respectively, consistent with the published affinity of the high molecular weight (type III) TGF-β1 binding site, betaglycan (Andres *et al.*, 1989).

Identification of Membrane Receptors by Covalent Cross-Linking. The cell-surface binding sites for ^{125}I-TGF-β1 have been studied extensively by radioligand cross-linking studies, and in SMC (Goodman and Majack, 1989), like many cells, the ^{125}I-TGF-β1 is found as three distinct complexes of > 200 kDa (type III), 85 kDa (type II), and 65 kDa (type I). The type III receptor has been identified as a membrane proteoglycan, betaglycan (Andres *et al.*, 1989). mRNA for the type II binding site, which exhibits homology to transmembrane serine/threonine kinases, has been identified recently in rat SMC (Lin *et al.*, 1992). To determine the type(s) of receptors down-regulated in the old SMC, radioligand cross-linking studies were performed. The majority of ^{125}I-TGF-β1 was associated with the type III (<200 kDa) and type II (85 kDa) binding sites (Figure 6). Old SMC exhibited reduced binding in all three receptor subtypes, and the type I and type II receptors were nearly undetectable. The faint band at ~35 kDa is frequently

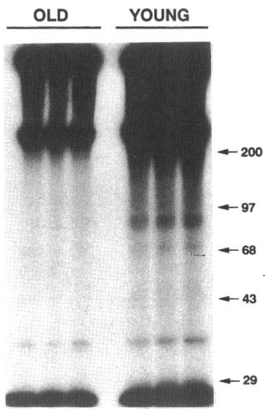

Figure 6. Analysis of ^{125}I-TGF-β1 binding sites on old and young SMC by chemical cross-linking. Binding of ^{125}I-TGF-β1 to old and young SMC was examined essentially as shown in Figure 5. Ligand-receptor interactions were covalently cross-linked with 250 μM BS^3 in PBS for 15 min before solubilization and 7% polyacrylamide gel electrophoresis under nonreducing conditions. Lanes reflect three separate cross-linkings of ^{125}I-TGF-β1 to old and young SMC. Control studies indicate that the >200-, 85-, and 65-kDa bands are not observed without BS^3 and are competed by cold TGF-β1.

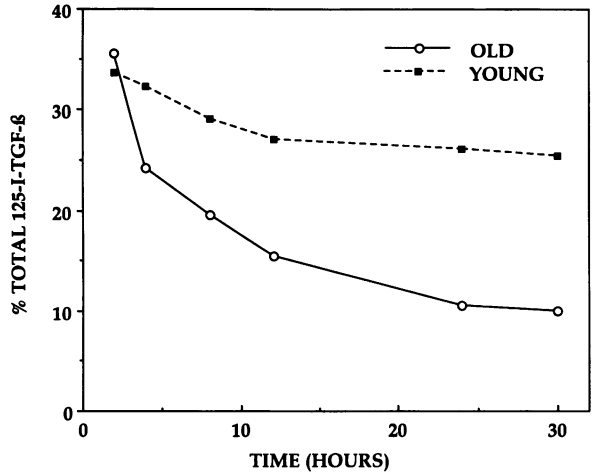

Figure 7. Age-related differences in the time-course of cellular processing of ^{125}I-TGF-β1. The cellular processing of ^{125}I-TGF-β1 at 37°C was examined in old and young SMC by determining cell-associated ^{125}I-TGF-β1 (NaOH solubilized cell monolayer) at the specified time points. The decreasing levels of cell-associated ^{125}I-TGF-β1 in the old SMC were associated with increasing TCA-soluble degradation products in the supernatant.

observed in ^{125}I-TGF-β1 preparations and, thus, is not a specifically cross-linked species. Control studies confirmed that the >200-, 85-, and 65-kDa binding sites were absent without the cross-linking agents and were reduced by an excess of cold ligand, consistent with published reports (Goodman and Majack, 1989).

The Effect of Aging on the Cellular Processing of TGF-β. Cell-surface binding of TGF-β1 is only one component of its biological action, which could be influenced by internalization and degradation. This was examined by measuring the binding and uptake of radiolabeled TGF-β1 at 37°C. The degradation of TGF-β1 was monitored by the appearance of TCA-soluble labeled peptides over time. The results indicate that despite the observed difference in cell-surface binding of ^{125}I-TGF-β1 at 4°C, the initial uptake of the ligand at 2 h was essentially equal at 37°C. However, old SMC rapidly degraded TGF-β, as evidenced by an increase in TCA-soluble radioactivity (11% of total counts at 2 h to 71% at 30 h) and a time-dependent decrease in cell-associated radioactivity (Figure 7). Young SMC maintained a much higher steady-state level (2.5-fold higher at 30 h) of cell-associated ^{125}I-TGF-β1 than did old SMC ($p < 0.05$) and degraded ^{125}I-TGF-β1 at a reduced rate (16% at 2 h to 55% at 30 h). Additional studies have determined that SMC degradation of ^{125}I-TGF-β1 is probably dependent on cellular uptake because no significant increase in TCA-soluble radioactivity is observed with cells treated at 4°C over periods as long as 120 h.

Age-Related Differences in the Production of Proteoglycan by SMC. Because the predominant SMC binding site for TGF-β1 appears to be the high molecular weight glycoprotein (type III), we examined the production of glycoprotein and protein in SMC with respect to age. ^{3}H-Glucosamine is a useful tracer for the glycosylation of glycoproteins and proteoglycans, whereas ^{3}H-proline is principally incorporated into collagen. The results, shown in Table 2, indicate that the incorporation of ^{3}H-glucosamine into soluble glycoprotein is significantly lower in old than young SMC ($p = 0.0009$). Insoluble matrix-associated glycoprotein was also markedly lower in old than young SMC ($p = 0.002$), leading to a twofold higher rate of total ^{3}H-glucosamine incorporation by young SMC. The magnitude and direction of this difference corresponds to previously reported differences in proteoglycan synthesis by gingival fibroblasts from human donors of differing ages (Barthold *et al.*, 1986). This appears to be specific to the incorporation of glucosamine because the incorporation of ^{3}H-proline into protein was not significantly different between the age groups.

DISCUSSION

A central paradox of the relationship between aging and atherosclerosis is that, in general, cells derived from aging subjects show a diminished proliferative capacity in vitro, yet aging individuals show an increased incidence of disorders with a proliferative component, ath-

Table 2. Age-related differences in proteoglycan and protein synthesis

	^{3}H-Glucosamine			^{3}H-Proline		
	Old	Young	p	Old	Young	p
Secreted	4 147.4 ± 72.1	6 780.2 ± 424.6	<0.001	4 578.3 ± 135.5	4 440.3 ± 482.1	NS
Insoluble	9 814.6 ± 112.6	20 410.0 ± 1 951.1	<0.05	22 656.2 ± 607.7	25 129.7 ± 2 443.6	NS
Total	13 659.9 ± 151.4	27 181.9 ± 2 152.6	<0.001	27 205.2 ± 710.0	29 569.9 ± 2 899.1	NS

The incorporation of isotopically labeled precursors (1 μCi/ml) was examined in SMC over a 24-h period followed by TCA precipitation of the supernatant (secreted) or dissolving the washed monolayer (insoluble). Data are expressed as mean ± SE. DPM per 1×10^5 cells (n = 4 per group). p values reflect the results of *t* tests (df = 6).

erosclerosis and cancer, in particular. Although there is extensive evidence that cells from elderly subjects show a shortened in vitro lifespan (Hayflick, 1965), there has been relatively little attention paid to the regulation of cell proliferation during the presenescent period. In fact, in this and other experimental systems, cells derived from aged subjects can be shown to proliferate at an increased rate before senescence (Holt and Yeh, 1988), an observation that is consistent with the much larger body of epidemiological data.

The present studies examine the cellular basis for an apparently nonneoplastic growth abnormality that is characterized by an age-related increase in the proliferation of vascular SMC under low-serum conditions. In the presence of normal cell culture levels of serum, the old and young SMC proliferate at similar rates and the old SMC show a shortened in vitro lifespan (McCaffrey, unpublished observations). However, under low-serum conditions, the old SMC demonstrate autocrine proliferation that can be suppressed by heparin (McCaffrey *et al.*, 1988). The present studies demonstrate that an important attribute of this proliferation is resistance to the autocrine antiproliferative effects of TGF-β1, a potent heparin-binding growth inhibitor for young SMC. The production of TGF-β1 by old SMC appears normal, as determined by Northern blot analysis and bioassay of serum-free media. Yet, the old SMC are essentially refractory to the antiproliferative effect of TGF-β1, as are SMC of increasing subpassage in vitro. This is the first example, to our knowledge, of loss of inhibition to TGF-β1 observed with in vivo aging.

Although the loss of TGF-β receptors and responsiveness has been observed in pathological states such as retinoblastoma (Kimchi *et al.*, 1988), these aging SMC represent one of the few instances of a nonneoplastic loss of TGF-β1 receptors. This may reflect a passive selection process for SMC variants with few receptors, which thereby confers a proliferative advantage in vivo or in vitro. Alternatively, reduced TGF-β binding and action has been reported during nonneoplastic differentiation of muscle cells (Ewton *et al.*, 1992), and thus it is possible that SMC may undergo changes in differentiation state with advancing age. The major binding site for TGF-β1 in SMC, the >200-kDa type III receptor, has been cloned (Lopez-Casillas *et al.*, 1991) and analyzed (Cheifetz *et al.*, 1988) and appears to lack signal transducing ability, although it may serve important storage and regulatory functions. Although the type II and type I receptors are more commonly found to vary with TGF-β actions (Boyd *et al.*, 1990; Howe *et al.*, 1990; Laiho *et al.*, 1990), the relationship of cell-surface receptors to TGF-β function remains uncertain. Epithelial cell variants with resistance to the antiproliferative effect of TGF-β1 have been generated in vitro by chemical mutagenesis, and these variants can express a normal or an altered pattern of cell-surface receptors (Boyd and Massagué, 1989; Howe *et al.*, 1990). Thus, changes in cell surface receptors are neither necessary nor sufficient for resistance to TGF-β, suggesting that postreceptor signaling is equally important. Preliminary evidence in our laboratory suggests that other responses to TGF-β1, such as increased collagen synthesis, could remain intact in the old SMC, further emphasizing the possible importance of discrete postreceptor signaling pathways.

In the present case, the resistance to TGF-β1 is also characterized by an increased ability to actively degrade TGF-β1, possibly suggesting a different intracellular processing of TGF-β1. Related studies indicate that an important effect of heparin, one of the soluble forms of cellular proteoglycan, and related compounds such as fucoidan, is to bind and protect TGF-β1 from scavenging proteins such as α_2-macroglobulin (McCaffrey *et al.*, 1989; McCaffrey *et al.*, 1992a) and to suppress proteolysis and intracellular degradation (McCaffrey and Falcone, unpublished data). Thus, the reduced surface binding and rapid cellular metabolism of TGF-β, possibly due to the loss of protection by endogenous sulfated polysaccharides, may be an important factor in the excessive proliferation of old SMC. In related studies, we were unable to identify an active inhibition (i.e., neutralizing ligand or specific protease) that would neutralize the action of TGF-β1 in old SMC, which favors the interpretation that membrane binding and intracellular processing, or additional postreceptor signaling events, are important determinants of TGF-β1 action in the SMC environment. Combined, the current evidence indicates that resistance to the antiproliferative effects of TGF-β1, possibly by altered cellular processing, may be an important component of the dysregulated growth that is observed in aging vascular smooth muscle cells. Future studies will examine the influence of receptor and postreceptor signaling as mechanisms for this resistance.

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REFERENCES

Andres, J.L., Stanley, K., Cheifetz, S., and Massague, J. (1989). Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-β. J. Cell Biol. *109*, 3137–3145.

(1976). Atherosclerosis of the aorta and coronary arteries in five towns. Bull. World Health Organization *53*, 485–645.

Barthold, M.P., Boyd, R.R., and Page, R.C. (1986). Proteoglycans synthesized by gingival fibroblasts derived from human donors of different ages. J. Cell. Physiol. *126*, 37–46.

Boyd, F.T., Cheifetz, S., Andres, J., Laiho, M., and Massague, J. (1990). Transforming growth factor-β receptors and binding proteoglycans. J. Cell Sci. Suppl. *13*, 131–138.

Boyd, F.T., and Massagué, J. (1989). Transforming growth factor-β inhibition of epithelial cell proliferation linked to the expression of a 53-kDa membrane receptor. J. Biol. Chem. *264*, 2272–2278.

Cheifetz, S., Andres, J.L., and Massagué, J. (1988). The transforming growth factor-β receptor type III is a membrane proteoglycan. Domain structure of the receptor. J. Biol. Chem. *263*, 16984–16991.

Danielpour, D., Dart, L.L., Flanders, K.C., Roberts, A.B., and Sporn, M.B. (1989). Immunodetection and quantitation of the two forms of transforming growth factor-β (TGF-β1 and TGF-β2) secreted by cells in culture. J. Cell. Physiol. *138*, 79–86.

Dartsch, P.C., Voisard, R., Bauridel, G., Hfling, B., and Betz, E. (1990). Growth characteristics and cytoskeletal organization of cultured smooth muscle cells from human primary stenosing and restenosing lesions. Arteriosclerosis *10*, 62–75.

Derynck, R., Jarrett, J.A., Chen, E.Y., and Goeddel, D.V. (1986). The murine transforming growth factor-β precursor. J. Biol. Chem. *261*, 4377–4379.

Ewton, D.Z., Spizz, G., Olson, E.N., and Florini, J.R. (1992). Decrease in transforming growth factor-β binding and action during differentiation in muscle cells. J. Biol. Chem. *263*, 4029–4032.

Goodman, L.V., and Majack, R.A. (1989). Vascular smooth muscle cells express distinct transforming growth factor-β receptor phenotypes as a function of cell density in culture. J. Biol. Chem. *264*, 5241–5244.

Gown, A.M. (1992). Cell type and cell state specific antibodies in the analysis of early lesions of human atherosclerosis. Am. J. Hypertens. *5* (suppl), 114S–117S.

Hariri, R.J., Alonso, D.R., Hajjar, D.P., Coletti, D., and Weksler, M.E. (1986). Aging and atherosclerosis. I. Development of myointimal hyperplasia after endothelial injury. J. Exp. Med. *164*, 1171–1178.

Hariri, R.J., Hajjar, D.P., Coletti, D., Alonso, D.R., Weksler, M.E., and Rabellino, E. (1988). Aging and atherosclerosis. Cell cycle kinetics of young and old arterial smooth muscle cells. Am. J. Pathol. *131*, 132–136.

Hayflick, L. (1965). The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. *37*, 614–636.

Holt, P.R., and Yeh, K.Y. (1988). Colonic proliferation is increased in senescent rats. Gastroenterology *95*, 1556–1563.

Howe, P.H., Cunningham, M.R., and Leof, E.B. (1990). Inhibition of mink lung epithelial cell proliferation by transforming growth factor-β is coupled through a pertussis-toxin-sensitive substrate. Biochem. J. *266*, 537–543.

Kimchi, A., Wang, X.F., Weinberg, R.A., Cheifetz, S., and Massague, J. (1988). Absence of TGF-β receptors and growth inhibitory responses in retinoblastoma cells. Science *240*, 196–198.

Laiho, M., Weis, M.B., and Massague, J. (1990). Concomitant loss of transforming growth factor (TGF)-β receptor types I and II in TGF-β resistant cell mutants implicates both receptor types in signal transduction. J. Biol. Chem. *265*, 18518–18524.

Lin, H.Y., Wang, X.F., Ng-Eaton, E., Weinberg, R.A., and Lodish, H.F. (1992). Expression cloning of the TGF-β Type II receptor, a functional transmembrane serine/threonine kinase. Cell *68*, 775–785.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S., and Massague, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-β receptor system. Cell *67*, 785–795.

Majack, R.A., Majesky, M.W., and Goodman, L.V. (1990). Role of PDGF-A expression in the control of vascular smooth muscle cell growth by transforming growth factor-β. J. Cell Biol. *111*, 239–247.

Masters, D.B., Griggs, C.T., and Berde, C.B. (1992). High sensitivity quantification of RNA from gels and autoradiograms with affordable optical scanning. BioTechniques *12*, 902–911.

McCaffrey, T.A., Falcone, D.J., Borth, W., Brayton, C.F., and Weksler, B.B. (1992a). Fucoidan is a novel, non-anticoagulant inhibitor of intimal hyperplasia. Biochem. Biophys. Res. Commun. *184*, 773–781.

McCaffrey, T.A., Falcone, D.J., Brayton, C.F., Agarwal, L.A., Welt, F.P., and Weksler, B.B. (1989). Transforming growth factor-β activity is potentiated by heparin via dissociation of the transforming growth-β/α_2-macroglobulin inactive complex. J. Cell Biol. *109*, 441–448.

McCaffrey, T.A., Falcone, D.J., and Du, B. (1992b). Transforming growth factor-β is a heparin-binding protein: identification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-β1. J. Cell. Physiol. *152*, 430–440.

McCaffrey, T.A., Nicholson, A.C., Szabo, P.E., Weksler, M.E., and Weksler, B.B. (1988). Aging and arteriosclerosis: the increased proliferation of arterial smooth muscle cells isolated from old rats is associated with increased platelet-derived growth factor-like activity. J. Exp. Med. *167*, 163–174.

Miyazono, K., Yuki, K., Takaku, F., Wernstedt, C., Kanzaki, T., Olofsson, A., Hellman, U., and Heldin, C.H. (1990). Latent forms of TGF-β: structure and biology. Ann. NY Acad. Sci. *593*, 51–58.

Nikol, S., Isner, J.M., Pickering, J.G., Kearney, M., Leclerc, G., and Weir, L. (1992). Expression of transforming growth factor-β1 is increased in human vascular restenosis lesions. J. Clin. Invest. *90*, 1582–1592.

Spagnoli, L.G., Orlandi, A., Mauriello, A., Santeusanio, G., De Angelis, C., Lucreziotti, R., and Ramacci, M.T. (1991). Aging and atherosclerosis in the rabbit. 1. Distribution, prevalence and morphology of atherosclerotic lesions. Atherosclerosis *89*, 11–24.

Sporn, M.B., Roberts, A.B., Wakefield, L.M., and Crombrugghe, B. (1987). Some recent advances in the chemistry and biology of transforming growth factor-β. J. Cell Biol. *105*, 1039–1045.

Stemerman, M.B., Weinstein, R., Rowe, J.W., Maciag, T., Fuhro, R., and Gardner, R. (1982). Vascular smooth muscle cell growth kinetics in vivo in aged rats. Proc. Natl. Acad. Sci. USA *79*, 3863–3866.

Wakefield, L.M., Thompson, N.L., Flanders, K.C., O'Connor-McCourt, M.D., and Sporn, M.B. (1988). Transforming growth factor-β: multifunctional regulator of cell growth and phenotype. Ann. NY Acad. Sci. *551*, 290–298.