Expression of Transforming Growth Factor-β1 Is Increased in Human Vascular Restenosis Lesions

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Abstract

Human atheromata obtained in vivo were used to test the hypothesis that transforming growth factor-β1 plays a role in the development of vascular restenosis.

We analyzed 28 specimens from patients with primary atherosclerotic or restenotic lesions; 26 of these were obtained by directional atherectomy and 2 at the time of coronary bypass surgery. Seven control tissues included operatively excised segments of human internal mammary artery, myocardium, and unused portions of vein graft obtained intraoperatively. From these 35 specimens, 210 sections were examined using in situ hybridization. Measurement of silver grains/nucleus disclosed that expression of transforming growth factor-β1 mRNA was highest in restenotic tissues ($P < 0.001$ vs. primary atherosclerotic tissues) and lowest in nonatherosclerotic (control) tissues. In cultures of human vascular smooth muscle cells grown from explants of internal mammary artery, expression of mRNA for transforming growth factor-β1 was significantly greater in subconfluent than in confluent smooth muscle cells ($P = 0.05$). Transforming growth factor type-β III receptor was expressed in cell cultures and undetectable in the tissue specimens. Sections taken adjacent to those studied by in situ hybridization were examined by immunohistochemistry using antibodies against transforming growth factor-β1 and α-actin (as a marker for smooth muscle cells) and disclosed transforming growth factor-β1 in smooth muscle cells present in these sections.

These findings are consistent with the concept that transforming growth factor-β1 plays an important role in modulating repair of vascular injury, including restenosis, after balloon angioplasty. (*J. Clin. Invest.* 1992. 90:1582–1592.) Key words: atherosclerosis • smooth muscle cell proliferation • in situ hybridization • cell culture • immunohistochemistry

Introduction

Cell division and migration of vascular smooth muscle cells are controlled by a network of interacting growth factors and receptors. It is widely accepted that smooth muscle cell proliferation is responsible for generation of restenotic lesions in vascular tissue (1–5). Although restenosis complicates balloon angioplasty in at least 40% of cases, identification of the events and specific factors responsible for restenosis remains elusive. A variety of growth factors has been described in vascular smooth muscle cells and/or atherosclerotic tissue and have therefore been inferred to play a role in the development of restenosis lesions (6–11); these include transforming growth factor-betas (TGF-βs)[1] (12–14), platelet-derived growth factors (PDGFs) and receptors (15, 16), fibroblast growth factors (12, 17), and insulin-like growth factor-1 (18).

The target of our investigation was TGF-β1, the originally described and generally most abundant isoform of TGF-β (19). Its cDNA has been cloned and its primary structure is well studied. It is a multifunctional peptide growth factor with a molecular weight of 25,000 in the configuration of a homodimer (20). In certain cell lines (21–24), TGF-β1 has been shown to bind preferentially to the TGF-β type III receptor (25, 26), although the signal transducing receptor for TGF-β is likely one of the low molecular weight receptors. TGF-β1 controls growth and differentiation of a wide spectrum of normal and neoplastic cells. For some mesenchymal cells, TGF-β1 has been found to be mitogenic in vitro and to stimulate wound healing in vivo (27). In contrast, TGF-β1 has been shown to inhibit proliferation of endothelial cells (28–30), certain mitogenically stimulated B and T lymphocytes (31–33), as well as normal and tumor-derived epithelial cells (34–36), and to retard epithelial wound healing in vitro (12).

TGF-β1 has also been reported to alternatively promote or inhibit growth, depending on cell age, cell density, the presence of coexistent factors (37–39), and the concentration of TGF-β1 (13, 19, 40). TGF-β1 can cause hypertrophy of smooth muscle cells, such as that resulting from systemic hypertension (37), as well as stimulate production of elastin (41) and proteoglycan (42), both of which are common features of atherosclerotic plaque.

Finally, the interaction of TGF-β1 with other growth factors is fundamental to the issue of thrombus formation, and dissolution. Specifically, basic fibroblast growth factor stimulates the production of tissue plasminogen activator by endothelial cells (43), whereas TGF-β1 inhibits release of tissue plasminogen activator mediated by basic FGF (37).

While the results of these in vitro experiments suggested variable, including at times contradictory, functions for TGF-β1, three in vivo experiments yielded concordant findings regarding the role of TGF-β1 in the repair of arterial injury. Madri et al. (44), using the balloon-injured rat carotid artery model, found that at 10 wk after injury, the neointima of such arteries displayed increased staining of TGF-β1. Majesky et al. (45), using the same model, confirmed the finding of increased TGF-β1 in neointimal smooth muscle cells; demonstrated that antecedent transcripts for TGF-β1 were increased by 6 h after

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1. *Abbreviations used in this paper:* IHC, immunohistochemistry; ISH, in situ hybridization; NMMHC-A, nonmuscle myosin heavy chain isoform A; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.

balloon injury; and showed that infusion of recombinant TGF-β1 stimulated proliferation of vascular smooth muscle cells. More recently, Winokur et al. (14) have reported evidence supporting the role of TGF-β isoforms in restenosis.

These findings prompted us to investigate human atheromata obtained in vivo, and cultures of vascular smooth muscle cells from human explants for evidence of growth-related expression of TGF-β1 and TGF-β type III receptor mRNA and protein.

Methods

Patients. Atherosclerotic tissue specimens were obtained from 28 patients with coronary artery or peripheral vascular disease. 14 of these specimens were obtained from patients with no history of percutaneous revascularization and were therefore classified as primary. The remaining 14 lesions were obtained from sites previously treated by balloon angioplasty and were therefore classified as restenosis lesions. For restenosis lesions, average time from previous angioplasty to current tissue retrieval was 7.50±6.19 mo. In addition to the atherosclerotic tissue specimens, seven control tissues including myocardium, normal internal mammary artery, and unused portions of saphenous vein were retrieved intraoperatively. The source of each tissue specimen is listed in Table I.

Tissue retrieval. In 26 of 28 patients, atherosclerotic specimens were retrieved by directional atherectomy using the Simpson AtheroCath (Devices for Vascular Intervention, Redwood City, CA) according to previously described techniques (46, 47). In the two remaining patients, atherosclerotic specimens were obtained at the time of coronary artery bypass surgery.

Tissue preparation. For both in situ hybridization (ISH) and immunohistochemistry (IHC), tissues were immersed in ice-cold 4% paraformaldehyde (Fisher Scientific Co., Pittsburgh, PA) for 2 h within minutes after retrieval to minimize RNA degradation followed by immersion in 30% sucrose/PBS (Fisher) overnight at 4°C. After embedding in O.C.T. compound (Miles Scientific Div., Miles Laboratories, Inc., Elkhart, IN), tissues were snap frozen in liquid nitrogen, stored at −70°C and finally sectioned on a cryostat (Miles) into 7-μm thick sections. Sections for ISH were placed on glass slides which had been previously coated with gelatin-chromic alum and incubated at 42°C overnight. Slides for IHC were placed on poly-L-lysine coated glass slides and stored at −70°C.

Cell culture. Human smooth muscle cell cultures were initiated by outgrowth from explants of internal mammary artery retrieved during coronary artery bypass surgery. Identification of smooth muscle cells was confirmed by positive immunostaining for α-smooth muscle actin (Sigma Chemical Co., St. Louis, MO) and lack of staining for Factor VIII-related antigen (Signet Laboratories, Dedham, MA). Cells at the third passage were seeded onto fibronectin-coated slides (10 μg/cm^2) at a density of 3,000 cells per cm^2 and allowed to grow for three days in medium 199 supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY). They were then fixed in 4% paraformaldehyde for 6 min and stored at −70°C before the hybridization procedure.

Table I. Source of Specimens Used in this Study

	In situ hybridization	Immunohistochemistry
	Specimens (sections)	
Atherectomy		
Primary coronary	7 (42)	1 (4)
Primary peripheral	5 (30)	2 (8)
Restenotic coronary	9 (54)	1 (4)
Restenotic peripheral	4 (24)	3 (12)
Restenotic coronary bypass	1 (6)	1 (4)
Surgery		
Primary coronary	1 (6)	
Primary coronary bypass	1 (6)	1 (4)
Internal mammary artery	3 (18)	2 (11)
Saphenous vein	2 (12)	
Myocardium	2 (12)	
Total	35 (210)	11 (47)

Preparation of probes. Freeze-dried *Escherichia coli* HB101 containing a plasmid with the insert encoding the complete coding sequence of human TGF-β1 (48) and human TGF-α (49) were obtained from the American Type Culture Collection (Rockville, MD).

The A-isoform of human nonmuscle myosin heavy chain is apparently present in all cells and presumably required for normal cell function (50). As such it may be considered as a "housekeeping" gene; previous studies in our laboratory have specifically disclosed no differences in expression in cells from primary versus restenosis lesions (50). The plasmid containing the 1.0-kb 3′-end of nonmuscle myosin heavy chain isoform A (NMMHC-A), kindly provided by Dr. Leslie Leinwand (Albert Einstein College of Medicine, Bronx, NY) (51), was therefore used as a "control" gene.

TGF-β1 is only ~ 70% similar in sequence to TGF-βs 2 and 3, both of which have separate genes on separate chromosomes, as well as different promoter structures and regulatory elements (52). After preparation of the plasmid, the 700-bp EcoRI/PstI fragment of TGF-β1, encoding the 3′-end of the insert, was isolated and subcloned into the polylinker of pGEM-3Zf(−) vector (Promega Corp., Madison, WI).

Similarly the 930-bp EcoRI fragment of TGF-α and the 1.0-kb EcoRI/PstI fragment of NMMHC-A were subcloned into pGEM-3Zf(−).

TGF-β2 is a 1.25-kb SmaI fragment in pBluescript KSII+ and TGF-β3 is a 1.23-kb SmaI fragment in pBluescript KSII+ (53, 54). Both were a generous gift of Drs. Denhez and Sporn (National Cancer Institute, Bethesda, MD).

The human TGF-β type III receptor cDNA probe was a generous gift of Drs. Lin and Lodish (Whitehead Institute, Cambridge, MA). It is a 4.5-kb EcoRI fragment in pBluescript SK- (25).

All probes contain dual promoters, which were used to generate antisense and sense RNA transcripts, labeled with [^{35}S]UTP (sp act: 1,300 Ci/mmol) (NEN Research Products, Boston, MA).

In situ hybridization. The technique for ISH used in this study was modified from that described previously (55). Before hybridization, sections from the 35 tissue specimens indicated above were rehydrated in PBS and 0.1 M glycine/PBS (Fisher), and then serially incubated in 0.3% Triton X-100/PBS (Fisher); Proteinase K (Boehringer-Mannheim GmbH, Mannheim, Germany); 4% paraformaldehyde: 0.25% acetic anhydride in triethanolamine (0.1 M, pH 8.0) (Fisher); and finally equal parts of diionized formamide (Sigma) and 4× standard saline citrate (SSC = 0.15 M NaCl/0.015 M trisodium citrate).

Tissue sections and cultured cells, prepared as described above, were incubated with the cRNA probe (3 × 10^5 cpm of ^{35}S-labeled probe/10 μl hybridization buffer) in a moist chamber for 16 h overnight at 42°C. Hybridization buffer consisted of 50% formamide, 2 × SSC, 10% dextran sulfate, 0.25% BSA, 0.25% Ficoll 400, 0.25% polyvinylpyrolidone 360, 250 mM Tris pH 7.5, 0.5% sodium pyrophosphate, 0.5% SDS, 250 μg/ml denatured salmon sperm DNA, 200 μg/ml yeast tRNA, and 10 mM dithiothreitol (Fisher, Sigma, Boehringer).

After hybridization, tissues were washed with 4 × SSC, treated with 20 μg/ml ribonuclease A (RNase A) (Sigma) in 0.5 M NaCl/10 mM Tris, pH 8.0/1 mM EDTA to eliminate nonhybridized cRNA strands, and washed in 2 × SSC and 0.1 × SSC. After dehydration in 70, 95, and 100% alcohol containing ammonium acetate, autoradiography in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) was performed, developed after 7 d, and counterstained with hematoxylin and eosin.

Immunohistochemistry. A total of 11 of 35 specimens on which ISH had been performed were selected for IHC (Table I). IHC was performed on adjacent sections. Frozen 7-μm tissue sections, fixed in 4% paraformaldehyde, were evaluated with three primary antibodies.

A monoclonal murine antibody to smooth muscle α-actin (clone 1A4, Sigma) was used to identify smooth muscle cells. For detection of TGF-β1, we used two different polyclonal rabbit antibodies, referred to as anti-LC and anti-CC (kindly provided by Drs. Flanders and Sporn, National Cancer Institute, Bethesda, MD). Both were raised to the peptide corresponding to the first 30 amino acids of mature TGF-β1; in Bouin's-fixed paraffin sections (56, 57), however, anti-LC recognizes intracellular protein, whereas anti-CC recognizes extracellular protein. Because cross-reaction of anti-LC with TGF-β2 has been previously reported, similar cross-reactivity with other TGF-β isoforms cannot be ruled out with certainty (56). Sections stained without antibody were used as negative controls.

All staining procedures were conducted at room temperature. After immersion in PBS, sections were blocked with 1.5% normal goat serum for anti-TGF-β1 or with normal horse serum for anti-α-actin, incubated with IgG fractions of the antibody, washed extensively, and then incubated with biotinylated goat anti-rabbit for anti-TGF-β1 or with horse anti-mouse for anti-α-actin IgG and avidine-enzyme complex. Sections were stained with 3,3'-diaminobenzidine and hydrogen peroxide (Signet) and counterstained with hematoxylin only.

Tissue section analysis. All sections were examined by light microscopy under dark-field and bright-field conditions at various magnifications (4–500). In an attempt to identify cell types expressing TGF-β1 in plaque, the in situ autoradiographed slides were counterstained with hematoxylin and eosin and examined by light microscopy at higher magnification. In some cases serial sections were processed for immunohistochemistry using anti-α-actin as a marker for smooth muscle cells. Unmanipulated serial sections were counterstained with both hematoxylin and eosin and elastic-trichrome.

Sections prepared for ISH were examined quantitatively by hand-counting of silver grains overlying cell nuclei at a magnification of 500. For each specimen hybridized with the antisense probe, 20 nuclei were randomly chosen: only the silver grains overlying the cell nuclei were considered, to determine a grain-to-nucleus ratio. Sections with high background (more than two silver grains per area of the size of an average nucleus) were excluded. Results were expressed as mean±SEM for each specimen. Comparisons were made using a one-way analysis of variance with Scheffe's F test. Statistical significance was set at $P < 0.05$.

Results

In situ hybridization. Among human vascular smooth muscle cells studied in culture, expression of TGF-β1 in cells from confluent cultures was significantly lower than expression observed in subconfluent human cells (151±14 grains/nucleus vs. 176±42 grains/nucleus, $P = 0.05$) (Fig. 1, Table II).

Expression of TGF-β1 mRNA was detected in cells of both atherosclerotic and nonatherosclerotic tissue specimens. These cells were identified as smooth muscle cells using immunostaining with anti-α-actin. Expression was lowest in nonatherosclerotic tissues (5±3 grains/nucleus) (Table III). Specimens of primary atherosclerotic lesions tended to demonstrate slightly higher expression compared to nonatherosclerotic tissues (10±4 grains/nucleus), but this difference was not statistically significantly. Expression of TGF-β1 was maximum in restenotic tissues (25±8 grains/nucleus, $P < 0.001$ vs. primary atherosclerotic tissues) (Fig. 2, Table III).

Expression of TGF-β1 was consistently high in restenotic tissues (Figs. 3 and 4) and consistently low in primary atherosclerotic tissues, regardless of the source (coronary artery, coronary artery bypass graft, peripheral artery). The background for sense controls was on average more than one grain per nucleus, whereas the results obtained with antisense probes were consistently and significantly greater.

Messenger RNA for TGF-β2 was not expressed at detectable levels, either in vascular tissues or in cultured vascular smooth muscle cells. TGF-α, TGF-β3, and TGF-β type III receptor mRNA were expressed at low levels in cultured vascular smooth muscle cells, but were undetectable in tissue specimens (data not shown). Expression of the housekeeping gene NMMHC-A was similar ($P = ns$) among specimens from nonatherosclerotic internal mammary arteries (13±2 grains/nucleus), primary atherosclerotic arteries (9±1 grains/nucleus), and restenotic tissues (11±3 grains/nucleus). All these additional gene probes were used on adjacent sections of tissue specimens previously used for TGF-β1.

Immunohistochemistry. Anti-α-actin was used in adjacent sections to identify smooth muscle cells for ISH as well as IHC. The media of the nonatherosclerotic internal mammary artery and most cells of the atherosclerotic tissues showed positive staining indicating high content of smooth muscle cells.

Tissues examined for TGF-β1 anti-LC and anti-CC showed positive staining in cells identified as smooth muscle cells by application of anti-α-actin to adjacent tissue sections (Fig. 5). In paraformaldehyde-fixed sections, staining for TGF-β1 protein occurs in a perinuclear distribution; as a result, staining for both antibodies of TGF-β1 was predominantly intracellular among tissues studied in the current investigation. This contrasts with results reported previously (56) for Bouin's-fixed tissues, in which TGF-β1 has been recognized both intracellularly and extracellularly. There was again no difference, regardless of the source.

Discussion

TGF was first described in 1978 and originally termed "sarcoma growth factor" (58). Its ability to both change the cellular morphology of tumor cells and facilitate growth of such cells in soft agar resulted in its being renamed "transforming growth factor" (59). Subsequently it was learned that these dual functions in fact represented the combined action of the two factors known today as TGF-α and TGF-β (60). The latter was also found in many normal cells including liver, heart, brain, kidney, muscle, and platelets (27, 39, 61, 62) and has been shown to exist in at least five isoforms (37). cDNA cloning has demonstrated that TGF-β1 has 70% sequence identity to isoforms 2 and 3 (52). TGF-β1 acts through a unique receptor complex that has no known homology to other growth factor receptors, and is devoid of tyrosine kinase activity (27, 62). The existence of several isoforms of TGF-β1 that act differently with a number of TGF-β receptor subtypes has been inferred to constitute a flexible system for the regulation of tissue growth and differentiation (63). Among certain nonvascular cell lines, the TGF-β type III receptor has been observed as the preferential and most abundant receptor for TGF-β1 (21–24), although the receptor responsible for TGF-β1 signal transduction may well be one of the two low molecular weight receptors.

The current study was designed to examine the expression of the isoform TGF-β1. This isoform was chosen for three reasons: (*a*) TGF-β1 had been previously demonstrated in vascular smooth muscle cells (12–14); (*b*) it had been the most abundant of the five isoforms (19); and (*c*) its cDNA had been previously cloned (48). The TGF-β1 protein sequence is abso-

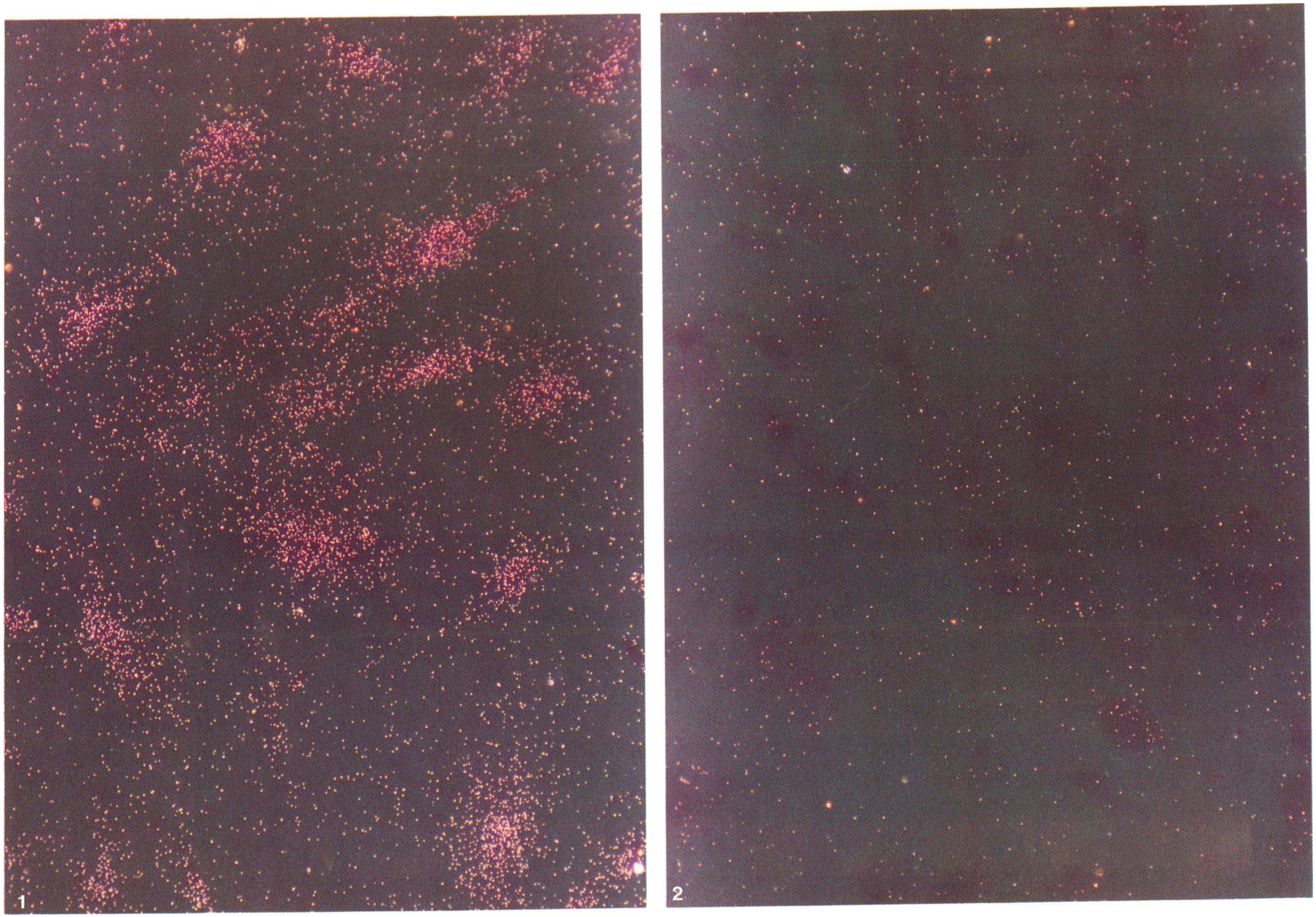

Figure 1. Dark-field microscopy: in situ hybridization with TGF-β1 of subconfluent cultures of human vascular smooth muscle cells. 1 = antisense and 2 = sense hybridization, ×50. Perinuclear clusters of silver grains are observed following antisense (1) but not sense (2) hybridization.

Table II. TGF-β1 mRNA among Cultured Vascular Smooth Muscle Cells

Vascular smooth muscle cells	TGF-β1 mRNA (grains/nucleus)	Significance
Subconfluent	176±42	$P < 0.05$
Confluent	151±14	

lutely conserved among several eucaryotic species, and is identical in man, monkeys, cows, pigs, and chickens (64), suggesting that any mutation might be detrimental or even lethal.

While the results of previous experiments performed in vitro have suggested variable mitogenic effects for TGF-β1 on cellular proliferation, in vivo studies reported from three independent laboratories (14, 44, 45) each yielded results pointing to a role for TGF-β1 in repair, including restenosis, after vascular injury.

To determine whether the in vivo observations reported in these animal model studies could be shown to extend to vascular disease, including restenosis, in humans, we investigated human atheromata. Access to such tissues has been facilitated by the advent of percutaneous directional atherectomy (46, 47). This particular device is unique among currently employed atherectomy instruments in that it retrieves tissue specimens essentially intact, sufficient to permit microscopic analyses (47). This is particularly advantageous for certain contemporary techniques such as ISH and IHC, both of which are less optimally performed on autopsy tissues due to the degradation of mRNA and loss of protein antigenicity, respectively.

The amount of tissue obtained by directional atherectomy is typically insufficient to detect mRNA by Northern blot. We have observed, however, that when the results of in situ hybridization are clearly positive, silver grains are preferentially associated with the nuclei. We have used this phenomenon to develop a quantitative scheme for analyzing the results of ISH by directly counting the number of silver grains per cell nucleus. Results in the present study have thus been expressed as an average number of grains/nucleus.

All tissues studied in the current investigation were found to express mRNA for TGF-β1; furthermore, expression of TGF-β1 in restenotic atherosclerotic tissues was significantly greater than that found in primary atherosclerotic tissue. Expression of TGF-β1 among cultures of human vascular smooth muscle cells was consistent with these observations: mRNA expression was higher among subconfluent, actively proliferating cells, than in those cultures in which further cell growth was inhibited by development of confluence. These findings thus suggest a relationship between the level of mRNA expression and degree of proliferation of human vascular smooth muscle.

The results of ISH must be interpreted to indicate relative levels of mRNA abundance, and may thus reflect levels of gene expression and/or stability of mRNA. The end result of the ISH procedure is a slide with dark silver grains in bright-field microscopy and white silver grains in dark-field microscopy, each of which indicates the presence of labeled probe. Washing procedures and RNase treatment remove most nonspecific binding. Even controls performed with sense probes, however, which would not be expected to hybridize, show a small background level of sliver grains which may vary depending upon the particular probe employed and the tissue inspected. We determined that subtraction of the average number of background grains/nucleus was unnecessary, because the primary results achieved statistical significance. Subtraction of the background grains/nucleus, however, would only be expected to increase the described differences.

ISH with the housekeeping gene NMMHC-A as control gene revealed no significant difference among nonatherosclerotic, primary atherosclerotic, and restenotic tissue specimens. In contrast, the increased expression observed among specimens from restenotic versus primary tissues is consistent with the notion that such increased expression of TGF-β1 mRNA is related to smooth muscle cell proliferation. The negative results obtained with probes for TGF-β2 and TGF-β3 in tissue specimens are consistent with observations made by Majesky et al. in balloon-injured rat carotid arteries studied by Northern blot analyses (45). Interestingly, TGF-β3 was expressed in confluent (i.e., nonproliferating) cultured smooth muscle cells, but was undetectable in proliferating smooth muscle cells. Negative ISH results obtained with a probe for TGF-α in tissue specimens confirmed previous results obtained by Wilcox et al. using operatively excised human carotid atherosclerotic plaques (65).

Adjacent sections of 11 specimens were examined with TGF-β1 anti-LC, TGF-β1 anti-CC, and anti-α-actin, and without antibody as a negative control. Although the two antibodies to TGF-β1 were both raised against amino acids 1–30 of the TGF-β1 protein, these antibodies appear to recognize different epitopes of the molecule in tissues preserved in Bouin's fixative before paraffin embedding (56, 57); anti-LC detects intracellular precipitates of TGF-β1, while anti-CC detects extracellular TGF-β1. Application of the two antibodies to the current series of specimens, all of which were prepared as paraformaldehyde-fixed frozen sections to optimize the results of in situ hybridization, indicated that the distribution of TGF-β1 was principally intracellular (Fig. 5). There was no difference with respect to the pattern of expression in restenotic vs. primary atherosclerotic lesions, regardless of the source. It is possible that the method of fixation used may have an important effect on the observations made. The effect of paraformaldehyde fixed-frozen sections on antibody reactivity has not been previously determined.

Examinations performed using IHC in sections adjacent to those examined by ISH indicated that the TGF-β1 protein was present in the same sites where its mRNA had been observed by ISH. That these were in fact vascular smooth muscle cells was shown by positive staining obtained with the anti-α-actin antibody.

The in vitro observations described in the present investigation are consistent with the aforementioned in vivo experiments involving TGF-β1 expression after vascular injury (44,

Table III. TGF-β1 mRNA among Tissue Specimens

Tissue specimens	Number of specimens	TGF-β1 mRNA (grains/nucleus)	Significance
Nonatherosclerotic	7	5±3	NS
Atherosclerotic (primary)	14	10±4	$P < 0.001$
Atherosclerotic (restenotic)	14	25±8	

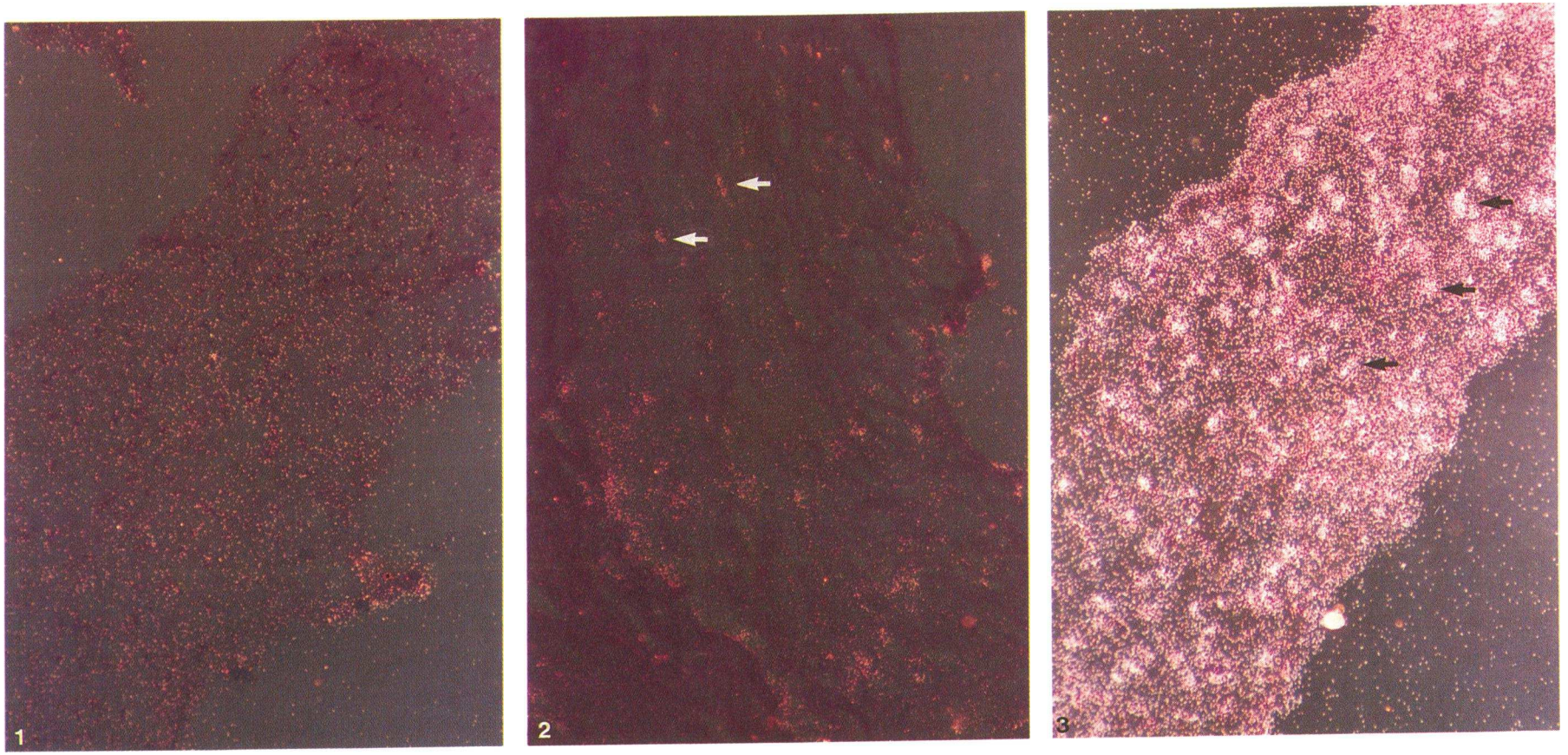

Figure 2. Dark-field microscopy: in situ hybridization with TGF-β1 probe. 1 = sense control, 2 = primary atherosclerotic coronary artery, and 3 = restenotic coronary artery (all $\times$50). In contrast to the sense control and primary coronary arterial lesion, the restenosis lesion contains perinuclear clusters of silver grains, which appear white (*arrows*) by dark-field microscopy.

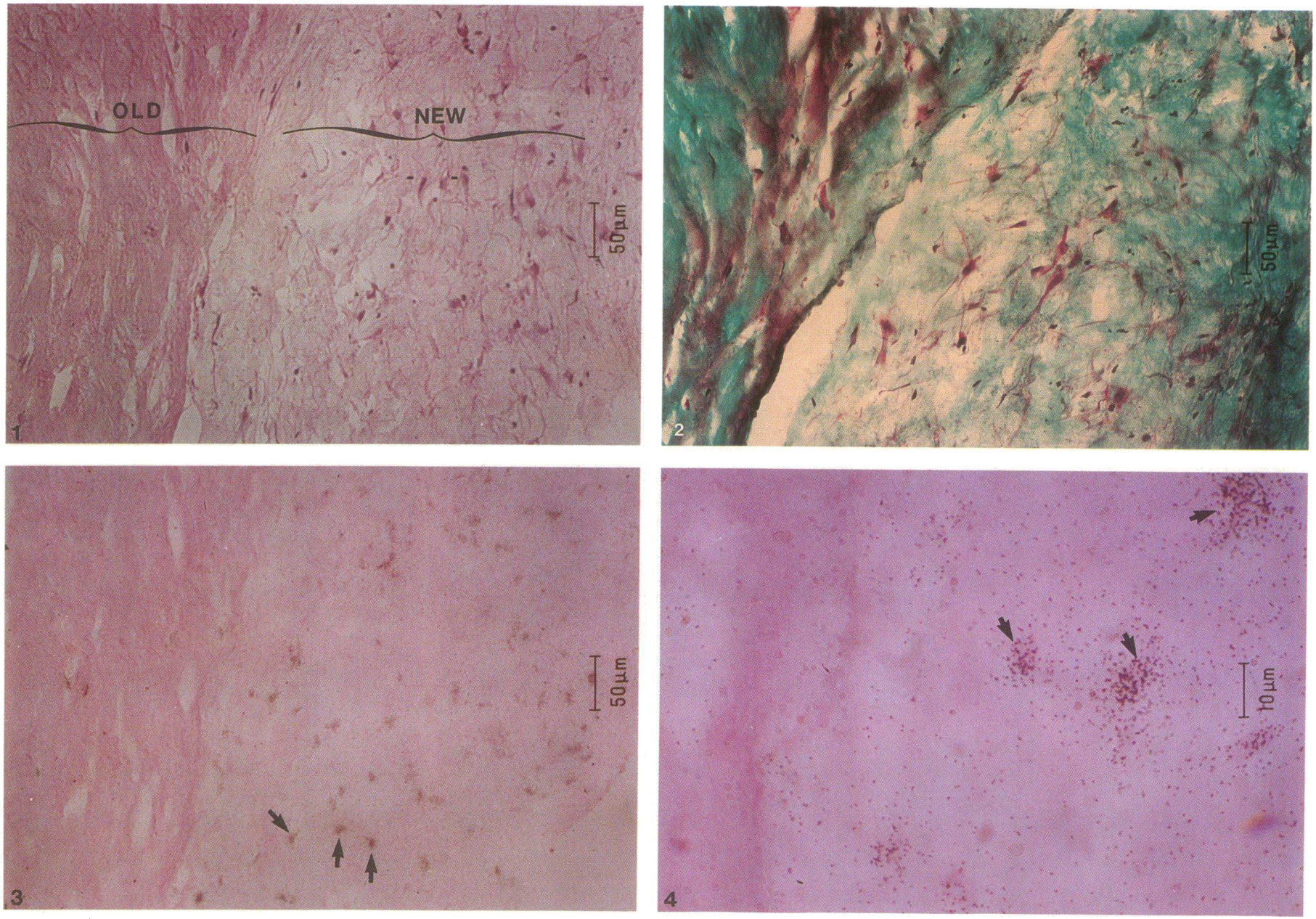

Figure 3. Bright-field microscopy: restenotic coronary atherectomy specimen. 1 = light microscopic section (hematoxylin and eosin, ×50) showing "bilayer" appearance typical of restenosis lesion: "old" plaque, hypocellular and consisting principally of collagen and ambiguous ground substance, is seen to left, while "new" plaque, consisting of loose connective tissue with greater cellularity, is seen to right. 2 = light microscopic section (elastic-tissue trichrome, ×50). 3 = in situ hybridization (×50) demonstrates multiple clusters of dark granules representing perinuclear silver grains, predominantly distributed throughout "new" plaque of "bilayer" specimen. 4 = in situ hybridization showing higher power (×250) view of cells positive for TGF-β1 mRNA near junction of "old" and "new" plaque.

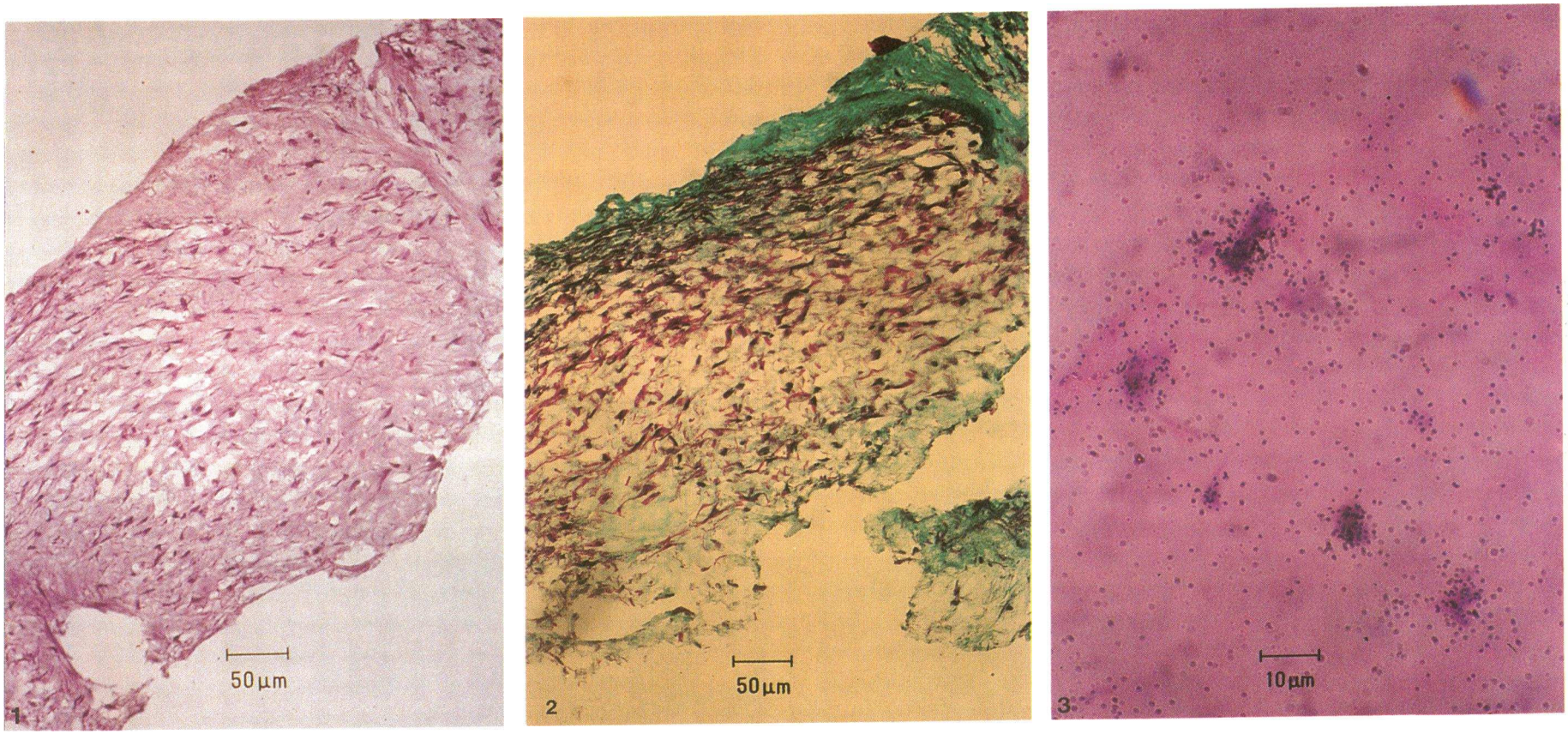

Figure 4. Bright-field microscopy: restenotic coronary artery specimen. 1 = light microscopic examination (hematoxylin and eosin, ×50) shows hypercellular plaque. 2 = elastic-tissue trichrome stained section of identical field at same (×50) showing loose connective tissue, typical of restenosis lesion. 3 = in situ hybridization (×250) showing perinuclear clusters of silver grains, indicative of TGF-β1 mRNA positivity.

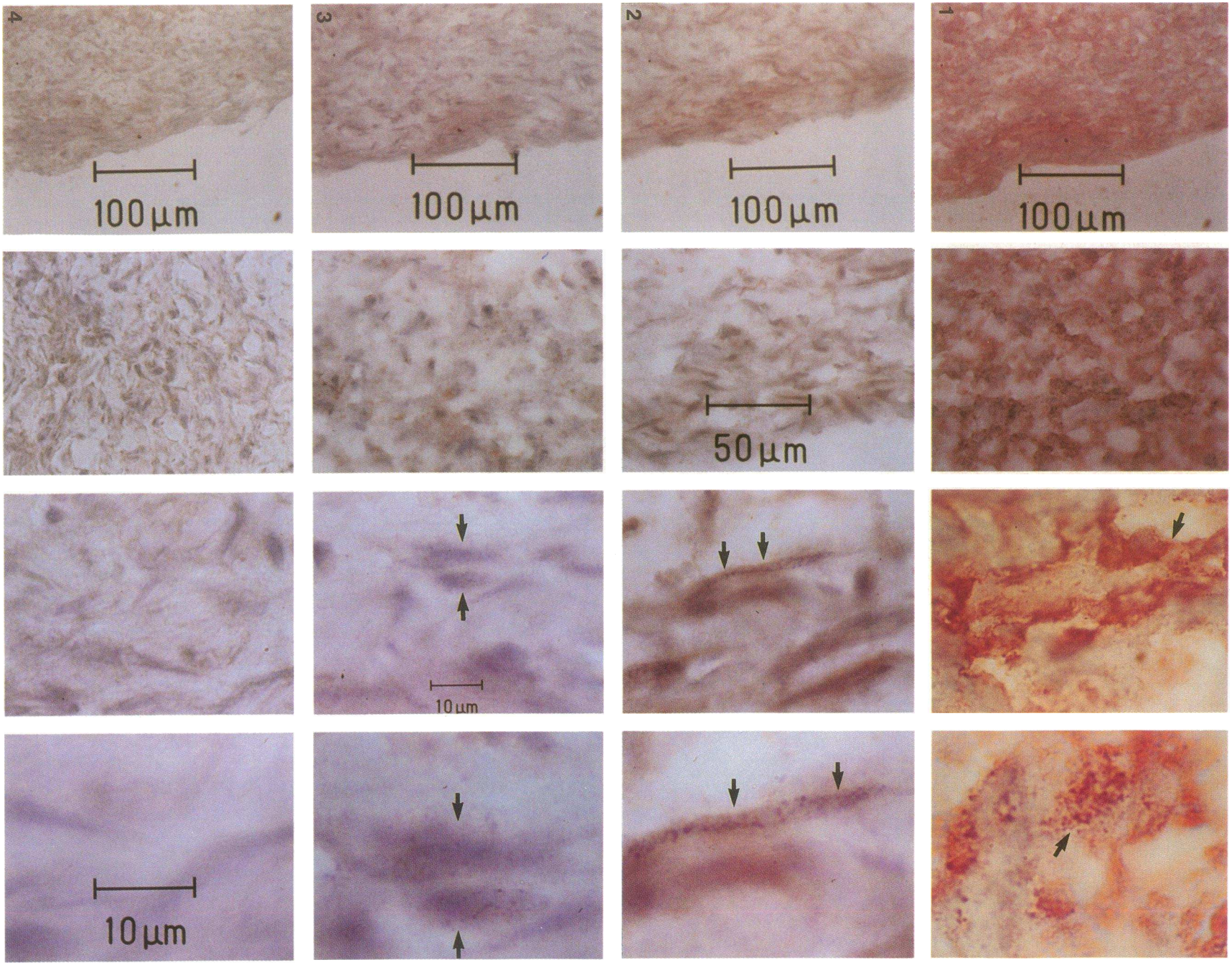

Figure 5. Immunohistochemistry in restenotic peripheral vascular lesion. Adjacent sections stained with anti-α-actin (line *1*); TGF-β1 anti-LC (line *2*); TGF-β1 anti-CC (line *3*); and negative control (line *4*). For each line, magnification (from left to right) is 50, 100, 250, and 500. Positive immunostaining appears as brown granules. Note the nuclear staining (*arrows*).

45). Majesky et al. used balloon angioplasty to produce focal injury in the rat carotid artery, and found that production of TGF-β1 was increased by 6 h and reached a maximum by 24 h during an observation period of 2 wk (45). Winokur et al. have recently reported further in vivo evidence in the rat carotid model supporting the role of TGF-β isoforms in restenosis (14).

Although TGF-β1 mRNA expression was significantly increased in restenotic tissues compared to primary and nonatherosclerotic tissues, and subconfluent cultures of human vascular smooth muscle cells compared to confluent cells, these preliminary findings can only be interpreted to suggest, but not prove, a role for TGF-β1 in the development of restenosis lesions. TGF-β1 has been previously reported to alternatively promote or inhibit growth, depending on cell age, cell density, the presence of coexisting factors (37, 38), and the concentration of TGF-β1 (13, 19, 40). For example, TGF-β1 inhibits the proliferation of smooth muscle cells in culture, but only at subconfluent densities, when proliferation is also mediated by PDGF (38). In confluent cells, on the other hand, TGF-β1 promotes proliferation of smooth muscle cells (37). The complex control of an autocrine PDGF loop has been found responsible for the bimodal proliferation induced by TGF-β1 in cultured smooth muscle cells, fibroblasts, and chondrocytes (40). At low concentrations TGF-β1 induces proliferation by stimulating PDGF-AA secretion, while at higher concentrations TGF-β1 inhibits proliferation by downregulation of the PDGF receptor α subunit (40). TGF-β1 also inhibits growth induced by fibroblast growth factor and epidermal growth factor in endothelial cells (28, 30, 37, 39), and modulates the synthesis of growth factor receptors (37).

Finally, it must be acknowledged that the presence of the TGF-β1 receptors has not yet been demonstrated in atherosclerotic tissues. This contrasts with the published finding of such receptors in immune killer cells (19), which, along with the finding of increased production of TGF-β1, has been interpreted to indicate an autocrine function for TGF-β1 in these cells. An autocrine function for TGF-β1 in vascular smooth muscle cells of atherosclerotic lesions would predict the presence of TGF-β receptors in these cells. Accordingly, we also performed ISH with a probe for the recently cloned TGF-β type III receptor (25), which is the preferential receptor for TGF-β1. In the current investigation, TGF-β type III receptor was detectable in cultured vascular smooth muscle cells at low levels, but in none of the tissue specimens. The proper interpretation of these findings remains uncertain, because cellular mechanisms of TGF-βs are still unknown (26). It is possible that in vascular tissues, the type III receptor is not the preferred or predominant receptor for TGF-β1. It is also possible that receptor preference for TGF-β1 may be altered under pathologic conditions, such as atherosclerosis and/or restenosis, and under such conditions TGF-β1 may be overexpressed due to a nonresponding type III receptor; or finally, that TGF-β type III receptor mRNA may be present, but at levels too low to be detectable.

Further elucidation of these remaining questions, including whether TGF-β1 acts in an autocrine fashion, whether it has a primary role in governing vascular smooth muscle cell proliferation, and how it interacts with other growth factors implicated to play a role in restenosis, may have important implications for gene and/or antisense therapy designed to inhibit restenosis following percutaneous revascularization.

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