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Active Macrophage-Associated TGF-β Co-Localizes with Type I Procollagen Gene Expression in Atherosclerotic Human Pulmonary Arteries

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Vascular remodeling in adult atherosclerotic pulmonary arteries is characterized by discrete areas of neointimal smooth muscle cell extracellular matrix gene expression in close proximity to non-foamy macrophages, suggesting regulation by local macrophage-associated factors. The purpose of these studies was to begin addressing the role of putative macrophage-associated factors such as transforming growth factor-β (TGF-β), by determining the spatial relationship between TGF-β and neointimal matrix gene expression in human atherosclerotic pulmonary arteries. For example, the participation of TGF-β in vascular remodeling could be inferred by its co-localization with non-foamy macrophages in areas of active matrix synthesis. In situ hybridization and immunohistochemistry demonstrated focal neointimal procollagen gene expression in close association with non-foamy but not foamy macrophages. Immunohistochemistry with isoform-specific anti-TGF-β antibodies demonstrated all three isoforms of TGF-β associated with non-foamy macrophages, but foamy macrophages were not immunoreactive. Neointimal and medial smooth muscle cells stained lightly. In contrast, intense TGF-β immunoreactivity was also associated with medial smooth muscle cells in normal nonremodeling vessels. Immunohistochemistry with antibodies specific for latent TGF-β was similar to immunohistochemistry for mature TGF-β in both remodeling and nonremodeling vessels. Finally, using an antibody specific for active TGF-β_1, immunoreactivity was only seen in non-foamy neointimal macrophages but not in foamy macrophages or medial smooth muscle cells from hypertensive or normal vessels. These observations suggest non-foamy macrophages may participate in modulating matrix gene expression in atherosclerotic remodeling via a TGF-β-dependent mechanism. (Am J Pathol 1995, 146:1140–1149)

The histological changes occurring in pulmonary hypertension are well described. [1-3] In the smaller muscular arteries, the pathological changes include medial hypertrophy, intimal proliferation, and fibrosis, as well as more complex changes such as the plexiform lesion. In contrast, the hypertensive elastic vessels become atherosclerotic, developing a thick neointima composed of smooth muscle cells, extracellular matrix, and inflammatory cells. Structural remodeling in the muscular arteries leads to increased pulmonary vascular resistance, whereas remodeling in the elastic pulmonary arteries decreases their compliance; both situations lead to increased right ventricular impedance and eventual right ventricular failure.[4] Thus, rational therapies based on intervening in the pathogenesis of primary pulmonary hypertension require defining the underlying mechanism(s) of vascular remodeling in both the small muscular arteries and the larger elastic vessels.

Recently, we demonstrated atherosclerotic changes in the large elastic pulmonary arteries from

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patients with severe unexplained pulmonary hypertension. [5] Immunohistochemical studies of these vessels showed elastin, type I collagen, and fibronectin proteins present diffusely throughout the neointima of hypertensive vessels. In contrast, active matrix protein synthesis occurred discretely, inasmuch as neointimal smooth muscle-like cells expressing extracellular matrix genes by *in situ* hybridization were found in focal clusters. Furthermore, matrix gene expression by these neointimal cells only occurred in association with small, non-foamy neointimal macrophages.[6] These observations suggested regulation of neointimal cell extracellular matrix gene expression by local macrophage-derived factors.

Macrophages may contribute to the formation or progression of atherosclerotic lesions by secreting factors, such as transforming growth factor-β (TGF-β), capable of stimulating extracellular matrix protein synthesis.[7,8] For example, exogenously administered TGF-β1 induces collagen and other extracellular matrix protein synthesis such as fibronectin and tropoelastin *in vitro* and *in vivo*.[9-15] In addition, several observations suggest extracellular matrix synthesis in remodeling tissues may be responding to endogenously produced TGF-β. For example, increases in TGF-β1 mRNA levels precede the development of pulmonary[16,17] and hepatic[18] fibrosis, TGF-β1 is associated with type I procollagen and fibronectin gene expression in idiopathic pulmonary fibrosis,[19] and neutralizing antibodies that block the action of TGF-β1 inhibit the accumulation of extracellular matrix in an animal model of glomerulonephritis.[20]

It is unclear, however, whether the increased extracellular matrix synthesis observed in vascular remodeling is modulated by TGF-β. Increases in neointimal steady-state levels of fibronectin and procollagen mRNA after carotid endarterectomy correlate better with the onset of neointimal formation than with increases in TGF-β1 mRNA.[21] Pulmonary artery TGF-β1 gene expression decreased, rather than increased as we predicted, in a hypoxic neonatal calf model of pulmonary hypertension compared with normoxic controls. TGF-β1 immunoreactivity and tropoelastin gene expression in the medial layer of these remodeling lobar pulmonary arteries also did not appear to co-localize.[22,23] Moreover, extracellular matrix gene expression in chronically remodeling vessels and after acute injury may not be responding to the same mediators. The temporal and spatial incongruities between TGF-β and vascular matrix gene expression, the inherent limitations of acute animal models of vascular remodeling, and the need to understand the pathogenic mechanisms of human disease before designing rational therapies, emphasize the need for studies in chronically remodeling human tissues.

Currently, three mammalian isoforms of TGF-β are described with similar structures and functions, although each has different promoters, suggesting each isoform has a unique role *in vivo*.[24] The co-localization of TGF-β protein with non-foamy macrophages in areas of matrix gene expression would provide a possible mechanism for modulation of vascular remodeling by non-foamy macrophages. The purpose of these studies was to begin addressing the role of macrophage-associated factors, such as TGF-β, in chronically remodeling hypertensive pulmonary arteries.

Materials and Methods

Tissue

Atherosclerotic hypertensive lobar pulmonary arteries were obtained from five patients, ages 20 to 39, undergoing single-lung transplant surgery for unexplained pulmonary hypertension at Washington University Medical Center. Normal lobar pulmonary arteries were obtained from five transplant donors. Immediately after surgical resection, pulmonary arteries were processed for histology, immunohistology, and *in situ* hybridization. Briefly, lungs were fixed overnight in 4% buffered formaldehyde at room temperature and embedded in paraffin. Hematoxylin and eosin and Verhoeff-van Gieson stains were applied to 5-μ thick sections from each tissue sample to demonstrate vascular structures.

In Situ *Hybridization*

In situ hybridization was performed essentially as previously described.[5,6] Type I procollagen (Hf677) cRNA probes were prepared with α[^{35}S]-uridine triphosphate (>1200 Ci/mmol, ICN Biochemicals, Irvine, CA) as described.[6] Hf677 is complementary to the C-terminal propeptide region of αI(I) procollagen mRNA.[25] [^{35}S]-labeled T66, a 500-nucleotide sense RNA probe transcribed from a bovine tropoelastin cDNA, served as a negative control. This RNA is 68% GC-rich and should have a propensity for nonspecific hybridization. Therefore, lack of *in situ* hybridization signal with this probe indicates appropriately stringent wash conditions. Hybridization solution containing 2.5×10^5 cpm [^{35}S]-labeled Hf677 antisense RNA probe was added to the processed sections and slides were incubated overnight at 55 C. After hybridization, slides were washed extensively under stringent conditions. To decrease background, slides

were incubated with 20 μg/ml RNase A to remove unhybridized probe. Washed slides were then processed for autoradiography.

Antibodies

TGF-β isoform-specific antibodies were prepared as described.[26] Briefly, peptides of each TGF-β isoform were synthesized, purified, and coupled to keyhole limpet hemocyanin for immunization of rabbits. For TGF-β1 and TGF-β2, peptides corresponding to residues 4–19 of the mature protein were used as immunogens. For TGF-β3, residues 9–20 were used. Specificity of each anti-peptide antibody was previously demonstrated.[26]

Antibodies were raised against the propeptide region of each latent TGF-β (LAP-TGF-β) isoform. Peptides of the precursor pro-region of each LAP-TGF-β isoform were synthesized, purified, and coupled to keyhole limpet hemocyanin for immunization of rabbits. Residues 81–102 were utilized for LAP-TGF-β1, residues 241–265 for LAP-TGF-β2, and residues 235–256 for LAP-TGF-β3. Rabbits were initially immunized with 250 μg of each peptide and subsequently boosted with 100 μg of peptide. Antibody titer was determined by enzyme-linked immunoadsorbent assay using the corresponding uncoupled peptide. Because a degree of homology is noted in the precursor region, each antiserum was screened for cross-reactivity with the nonimmunizing peptides. Animals demonstrating cross-reactivity were not used. The anti-LAP-TGF-β antibodies were purified by ammonium sulfate precipitation followed by peptide-affinity chromatography using the respective immunogenic peptide.

HAM56 is a mouse anti-macrophage monoclonal antibody that may also cross-react with endothelial cells (a generous gift of Dr. A. Gown, University of Washington, Seattle, WA).[27] Rabbit polyclonal anti-human TGF-β1 antibody[28] was kindly supplied by K. Flanders (National Institutes of Health, Bethesda, MD). This antibody recognizes active TGF-β1 in frozen tissue sections fixed in methanol/acetone[29] as well as in formalin-fixed, paraffin-embedded tissues (Dr. M. H. Barcellos-Hoff, personal communication).

Immunohistochemistry

Sections were prepared for immunoperoxidase staining as described.[5,30] Endogenous peroxidase was blocked with 0.3% (v/v) H_2O_2 in methanol for 1 hour at room temperature. Sections were treated with hyaluronidase (1 mg/ml) (Sigma Chemical Co., St. Louis, MO) for 20 minutes at 37 C. Nonspecific immunoglobulin binding sites were blocked with normal goat (TGF-β antibodies) or horse (HAM-56 antibody) serum. Sections were subsequently incubated overnight at 4 C with TGF-β isoform-specific antibodies (2.5 μg/ml) or HAM-56 (1:4000). Sections were incubated with the rabbit polyclonal anti-human TGF-β1 antibody (10 μg/ml) at room temperature for 4 hours. Species matched normal IgG or serum, respectively, served as negative controls. Sections were then incubated for 20 minutes with affinity-purified biotin-conjugated goat anti-rabbit or horse anti-mouse IgG (1:1600 dilution) (Vector Laboratories, Burlingame, CA), washed and incubated for 60 minutes with horseradish peroxidase-streptavidin (1:400 dilution) (Vector Laboratories). Immunoglobulin complexes were then visualized by incubation with 3,3′-diaminobenzidine (0.5 mg/ml in 50 mmol/L Tris-HCl, pH 7.4) in 0.3% H_2O_2. Sections were counterstained with Gill's hematoxylin, dehydrated, mounted in Permount, and examined by light microscopy.

Results

Previous sequential immunohistochemistry-*in situ* hybridization studies in three patients with hypertensive, atherosclerotic pulmonary arteries demonstrated small, non-foamy macrophages in close proximity to discrete clusters of neointimal smooth muscle-like cells expressing the genes for type I procollagen and fibronectin.[6] In the current study, *in situ* hybridization using hypertensive pulmonary arteries from five additional patients confirmed focal type I procollagen gene expression only by neointimal cells (Figure 1, A and B). Furthermore, immunohistochemical studies with serial sections of these tissues, using antibody HAM-56, again demonstrated small non-foamy macrophages in the same region as neointimal cells expressing the gene for type I procollagen (Figure 1C). As in our previous studies, neointimal cells near foamy macrophages did not express type I procollagen mRNA by *in situ* hybridization (Figure 2, A to C). Furthermore, extracellular matrix gene expression appeared confined to the neointima, given that no medial cells from hypertensive or normal pulmonary arteries expressed type I procollagen mRNA (data not shown). These observations again suggest that small, non-foamy neointimal macrophages participate in the local regulation of neointimal cell matrix gene expression.

Prerequisites for the participation of putative macrophage-derived factors, such as TGF-β, in the local regulation of extracellular matrix protein synthesis are 1) the presence of those factors in areas of

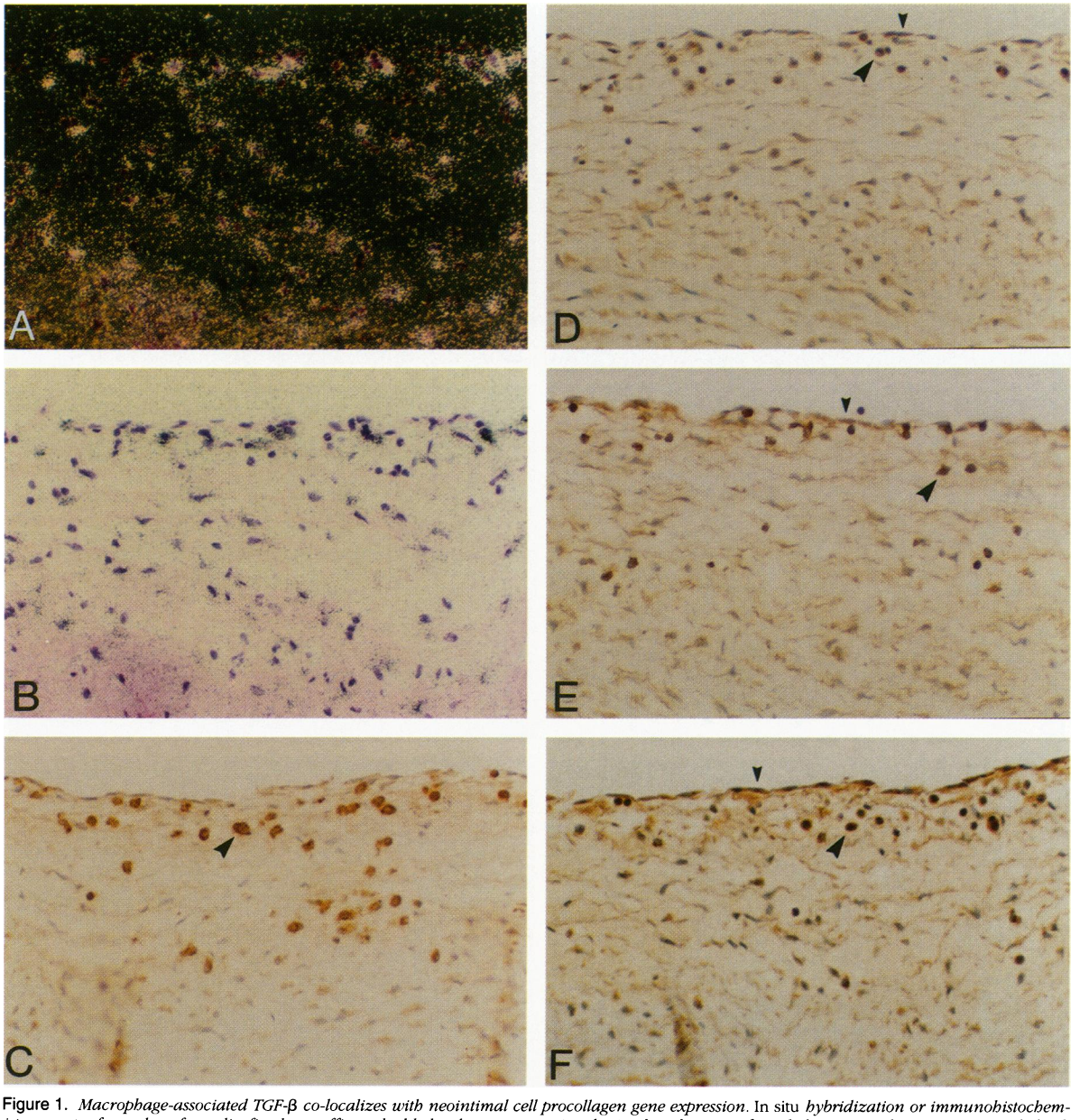

Figure 1. *Macrophage-associated TGF-β co-localizes with neointimal cell procollagen gene expression.* In situ *hybridization or immunohistochemistry was performed on formalin-fixed, paraffin-embedded pulmonary arteries obtained at the time of single-lung transplantation.* In situ *hybridization was performed with a procollagen α1(I) [^{35}S]-labeled cRNA probe* (**A** *and* **B**, *darkfield and brightfield, respectively*). *Macrophages would not be detected in A and B, because immunohistochemistry was not performed on these sections. Immunohistochemistry was performed on adjacent sections of the same tissue with antibodies to macrophages* (**C**), *TGF-β1* (**D**), *TGF-β2* (**E**), *and TGF-β3* (**F**). *Active procollagen gene expression by smooth muscle-like cells in this region of the neointima (medial layer not shown) occurs in close association with small, non-foamy macrophages that stain intensely for all three isoforms of TGF-β (large arrowheads) using antibodies directed against a sequence from each mature TGF-β. Endothelial cells lining the vascular lumen also stain intensely (small arrowheads). TGF-β immunoreactivity associated with neointimal smooth muscle cells is slightly greater than background although not as intense as with macrophages or endothelial cells. These results were consistently seen in all areas of active remodeling (defined by procollagen gene expression) in hypertensive pulmonary arteries from five separate patients. All tissues incubated with normal control IgG at concentrations equivalent to the specific antibodies were negative, and* in situ *controls were also negative. (Magnification ×200)*

active matrix synthesis, and 2) the association of those factors with non-foamy macrophages but not with foamy macrophages. Therefore, immunohistochemistry was performed with antibodies to all three mammalian isoforms of TGF-β on serial sections of actively remodeling atherosclerotic pulmonary arteries. Non-foamy macrophages in the same region as neointimal cells expressing procollagen mRNA stained intensely for all three isoforms of TGF-β (Figure 1, D to F). In contrast, foamy macrophages did not stain for TGF-β (Figure 2, D to F). Endothelial cells from hypertensive vessels also stained intensely

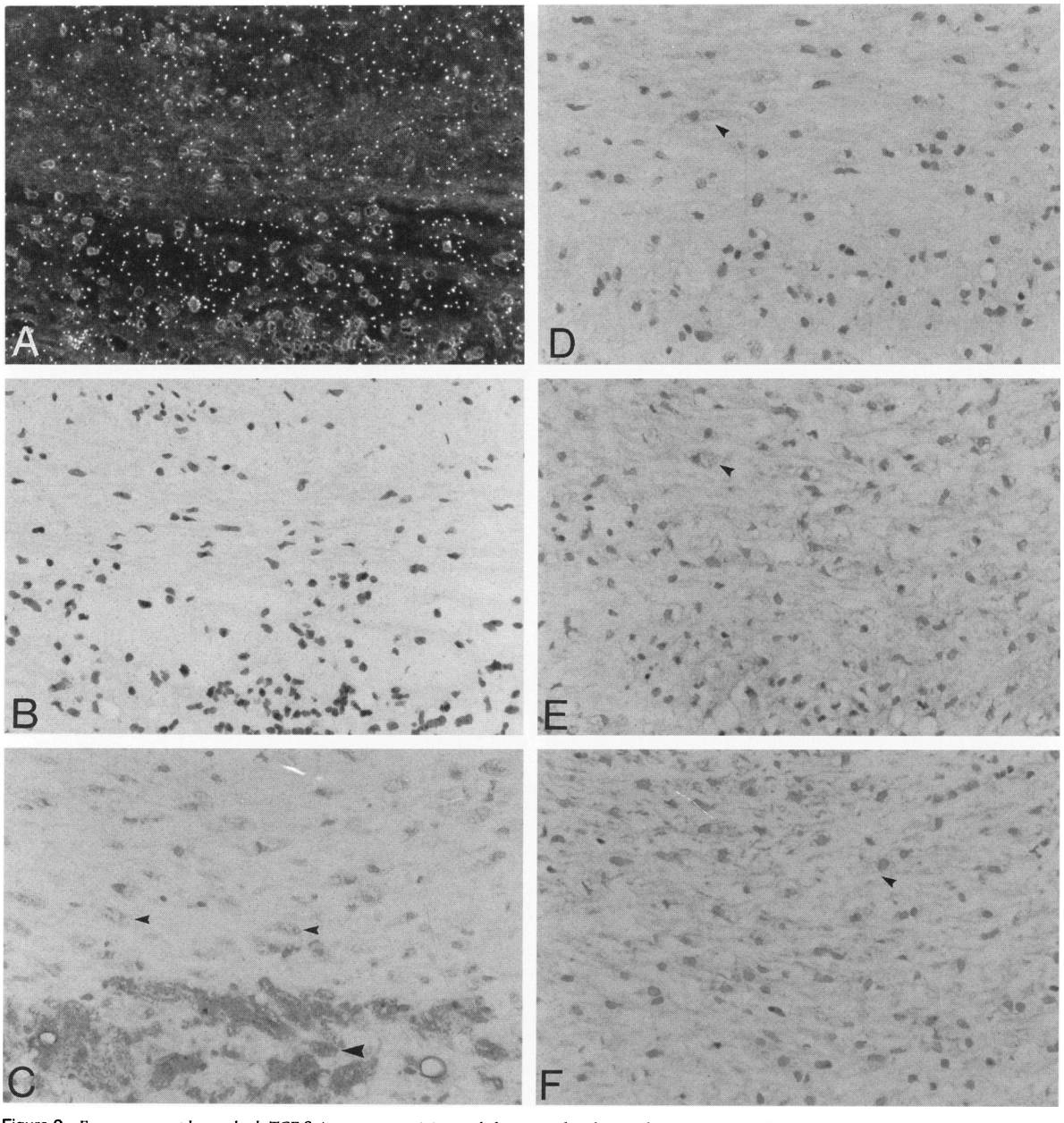

Figure 2. *Foamy macrophages lack TGF-β immunoreactivity and do not co-localize with neointimal cell procollagen gene expression.* In situ *hybridization and immunohistochemistry from a nonremodeling region of the same atherosclerotic pulmonary artery described in Figure 1. No procollagen gene expression is observed in this area of the neointima* (**A** *and* **B**). (*Small arrowheads*) *Representative neointimal smooth muscle cells. Only foamy macrophages* (*large arrowheads*) *are present* (**C**, *bottom*); *these do not stain for any isoform of TGF-β* (**D** *to* **F**) *using antibodies directed against a sequence from each mature TGF-β. The vascular lumen and medial layers are not shown but would be at the top and bottom of the figures, respectively.* (*Magnification,* ×*200*)

(Figure 1, D to F) but neointimal smooth muscle cells, medial smooth muscle cells, and cells within the adventitia only stained lightly. These results were seen consistently in hypertensive vessels from all five patients. That TGF-β was present only in non-foamy macrophages closely associated with cells actively expressing procollagen, but absent in foamy macrophages not associated with cells actively expressing procollagen, is consistent with the hypothesis that macrophage-associated TGF-β participates in regulating atherosclerotic vascular remodeling.

Immunohistochemistry also demonstrated, however, all three isoforms of TGF-β in normal, nonremodeling pulmonary arteries. In contrast to remodeling vessels where immunoreactivity was primarily macrophage-associated, immunoreactivity in normal vessels was associated with medial smooth muscle cells (Figure 3). As a result, the intensity of medial

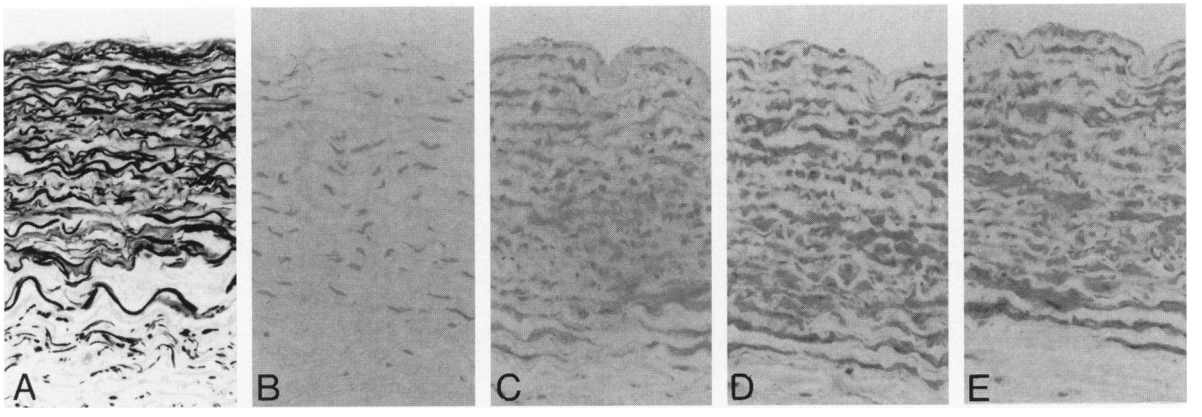

Figure 3. *TGF-β is present in normal nonremodeling pulmonary arteries. Normal pulmonary arteries were obtained from lung transplant donors and processed as described in the legend to Figure 1. Immunohistochemistry was performed with antibodies to TGF-β1* (**C**), *TGF-β2* (**D**), *and TGF-β3* (**E**). *Normal rabbit IgG served as a control* (**B**). *Vascular structures are demonstrated with an elastin-van Gieson stain* (**A**). *No active matrix gene expression is demonstrable in these vessels, although intense smooth muscle-associated immunoreactivity is seen for TGF-β2 and TGF-β3. TGF-β1 immunoreactivity is less intense compared with the other two isoforms.*

smooth muscle cell immunoreactivity for each isoform was greater in normal compared with hypertensive vessels when immunohistochemistry was performed under similar conditions. Immunoreactivity was most intense for TGF-β3 and least intense for TGF-β1 with equal concentrations of antibody. No immunoreactivity was observed with normal rabbit IgG.

TGF-β is produced and secreted by cells in a biologically inactive form composed of noncovalently associated latent and mature regions. The inactive form can be activated after secretion from the cell and is composed of a disulfide-linked dimer of mature regions only. The antibodies used for the studies described above, generated against a peptide sequence found in the mature region, cannot distinguish between the latent and mature forms of TGF-β because the latent TGF-β molecule is composed of both the latent and mature regions. The possibility that TGF-β immunoreactivity observed in normal, nonremodeling vessels represents latent TGF-β precursors was studied using antibodies specific for the latent form of TGF-β2 and TGF-β3 (see Materials and Methods). Immunohistochemistry with an antibody to latent TGF-β3 demonstrated immunoreactivity associated with medial smooth muscle cells from normal vessels in a pattern identical to immunoreactivity with antibody to mature TGF-β3 (Figure 4, A and B). Furthermore, the pattern of latent TGF-β3 immunoreactivity in hypertensive pulmonary arteries was similar to mature TGF-β3 immunoreactivity (Figure 4, C and D). Intense latent and mature TGF-β3 immunoreactivity was associated with non-foamy neointimal macrophages in areas of active neointimal procollagen gene expression, whereas only faint immunoreactivity was observed in macrophage-poor areas of the neointima (Figure 4, E and F) or in areas populated primarily by foamy macrophages (data not shown). Similar results in normal and hypertensive vessels were observed using an antibody to latent TGF-β2 (data not shown).

To determine whether active TGF-β co-localized with areas of active type I collagen gene expression, and conversely, whether active TGF-β was absent in nonremodeling vessels, immunohistochemistry was performed with an antibody that specifically recognizes active TGF-β1.[29] Previous studies had demonstrated that increased immunoreactivity with this antibody correlated with increased steady-state levels of TGF-β1 mRNA in normally developing neonatal pulmonary arteries.[23] In our current studies, active TGF-β1 immunoreactivity was found in non-foamy macrophages and endothelial cells from hypertensive arteries (Figure 5). Foamy macrophages and medial smooth muscle cells from hypertensive vessels, and endothelial cells and medial smooth muscle cells from normal vessels, were unreactive (data not shown). The presence of active TGF-β2 or TGF-β3 could not be determined because antibodies specific for the active forms of these growth factors are not available.

Discussion

Vascular remodeling in adult atherosclerotic elastic pulmonary arteries is characterized by discrete areas of neointimal smooth muscle cell matrix gene expression, suggesting regulation by local factors.[5,6] Although the factors responsible for inducing matrix gene expression in atherosclerotic lesions remain unknown, two observations suggest macrophages may be a focal source of those factors. First, non-foamy

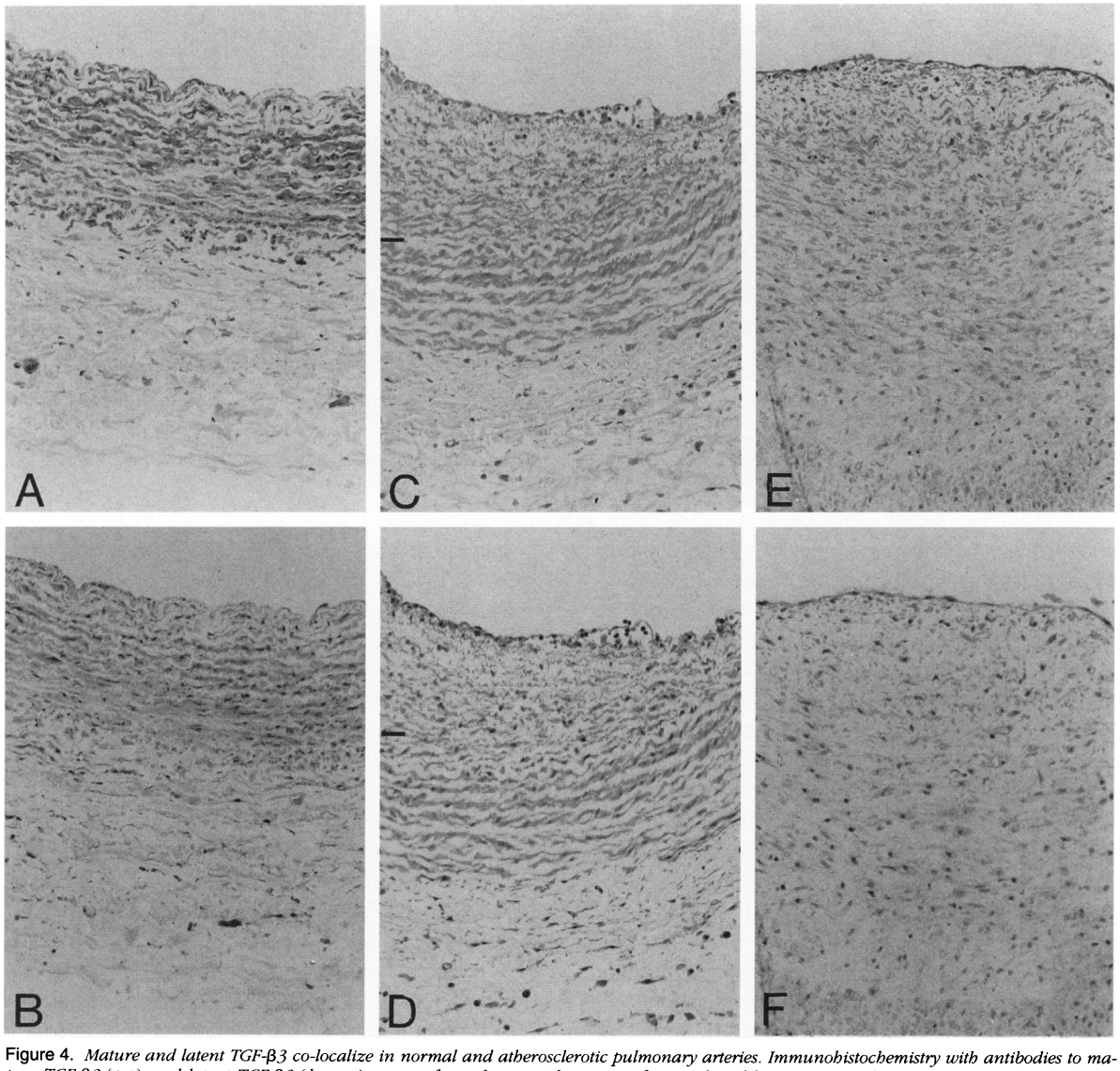

Figure 4. *Mature and latent TGF-β3 co-localize in normal and atherosclerotic pulmonary arteries. Immunohistochemistry with antibodies to mature TGF-β3 (top) and latent TGF-β3 (bottom) was performed on serial sections of normal and hypertensive pulmonary arteries. The pattern of mature and latent TGF-β3 immunoreactivity was similar in all vessels including nonremodeling normal pulmonary artery* (A *and* B), *an actively remodeling region of neointima from a hypertensive vessel* (C *and* D), *and a region of neointima from a hypertensive vessel without demonstrable procollagen gene expression* (E *and* F). *Similar immunohistochemical staining was observed with antibodies to mature and latent TGF-β2 (not shown).*

macrophages are found in close proximity to neointimal cells expressing matrix genes.[6] Second, several factors secreted by macrophages,[7,8] including TGF-β, are capable of stimulating extracellular matrix protein synthesis. Our observations that 1) neointimal non-foamy macrophages are closely associated with matrix gene expression and are also immunoreactive for all three isoforms of TGF-β (including active TGF-β1), and 2) that foamy macrophages that do not co-localize with matrix gene expression lack TGF-β immunoreactivity (including active TGF-β1), satisfy a necessary prerequisite for a role for TGF-β in atherosclerotic remodeling.

The presence of TGF-β immunoreactivity in non-remodeling normal large, elastic pulmonary arteries, requires explanation. The synthesis of active homodimeric TGF-β is complex and highly regulated. TGF-βs are synthesized as large precursor monomeric peptides, with the mature sequence contained within the C-terminal 112 amino acids.[31] Post-translational modification includes 1) disulfide-linked dimerization; 2) glycosylation (which appears required for secretion, since nonglycosylated TGF-β remains intracellular)[32-35]; and 3) proteolytic cleavage of the latent amino-terminal propeptide from the C-terminal mature protein, although they remain non-

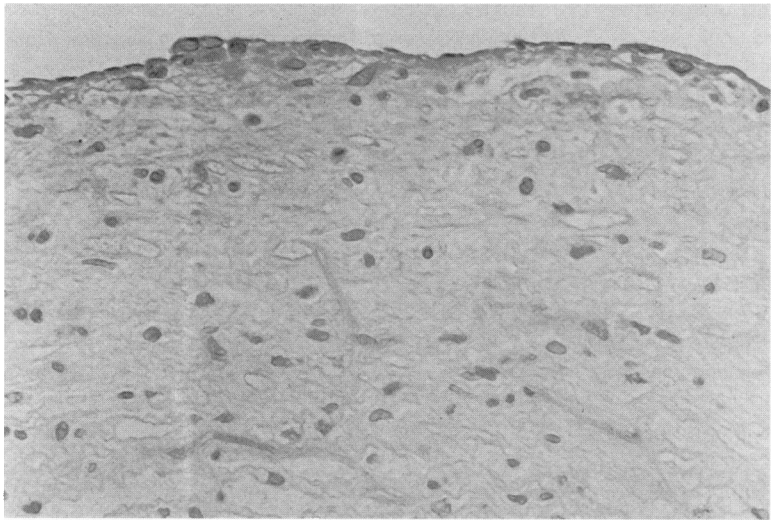

Figure 5. *Active TGF-β1 immunoreactivity is associated with non-foamy macrophages. Immunohistochemistry with anti-TGF-β1 antibody LC was performed on normal and atherosclerotic pulmonary arteries. Non-foamy macrophages and endothelial cells stain positively. Hematoxylin counterstaining was omitted to demonstrate more clearly TGF-β1 immunoreactivity. The vascular lumen is at the top of the figure. (Magnification ×200)*

covalently associated.[31,36] In addition, a large protein, latent TGF-β binding protein, is added to the complex, possibly to facilitate secretion, because TGF-β complexed with latent TGF-β binding protein is secreted from the cell more readily than noncomplexed TGF-β.[37] Once secreted, latent TGF-β may bind various extracellular matrix proteins[38-40] until activation and binding with specific target-cell receptors.[41] Latent TGF-β binding protein may function in these processes as well.[42]

One explanation, therefore, for the presence of TGF-β immunoreactivity in nonremodeling pulmonary vessels is that the antibodies identify a pool of latent TGF-β. The absence of immunoreactivity in the nonremodeling medial layers of normal and hypertensive arteries, using an antibody that specifically recognizes active TGF-β1, is consistent with this explanation. Furthermore, these observations are similar to immunohistochemical studies demonstrating latent TGF-β in normal murine mammary glands[29] and biochemical evidence for a pool of latent TGF-β in nonremodeling bone.[43,44] The step(s) at which processing of these latent TGF-β molecules ceases remains unknown, but if this hypothesis is correct these results suggest that post-translational modification, secretion, or activation of TGF-β could be important and regulated differences between remodeling and nonremodeling tissues. The presence of immunoreactive TGF-β in tissues, using antibodies that do not distinguish between mature and latent TGF-β, does not necessarily indicate activity or participation in normal or pathological processes. However, the close association between cells expressing type I procollagen mRNA and cells staining for TGF-β is consistent with the presence of active TGF-β in the neointima of hypertensive tissues.

Several observations suggest that foamy and nonfoamy macrophages have different functional phenotypes. For example, lipoprotein lipase is synthesized predominantly by the most lipid-laden foamy macrophages in human coronary atherosclerotic plaques.[45] Furthermore, immunoreactive PDGF-BB is only found in nonlipid-laden macrophages.[46] The presence of TGF-β immunoreactivity (and monocyte chemoattractive protein-1 and interleukin-8 immunoreactivity; M. Botney, unpublished observations) only in non-foamy macrophages further suggests functional differences between foamy and non-foamy macrophages. Whether the differences between foamy and non-foamy macrophages reflects a generalized differentiation of all non-foamy macrophages or selection of a unique subpopulation of non-foamy macrophages is unknown.

In summary, vascular remodeling is a very complex biological process of cellular proliferation and matrix production. The activity of many putative regulatory factors such as TGF-β found in atherosclerotic lesions may vary greatly depending upon the precise cellular, matrix, and cytokine milieu in which they are found. Our results do not prove extracellular matrix gene expression is directly modulated by TGF-β in remodeling tissues, but do indicate that a pool of latent TGF-β exists in nonremodeling arteries and that active TGF-β is present in actively remodeling vessels. These results underscore the importance of studying the regulation and processing of TGF-β *in vivo*.

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