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# Vascular Remodeling in Primary Pulmonary Hypertension

## *Potential Role for Transforming Growth Factor-β*

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***Active exogenous transforming growth factor-βs (TGF-βs) are potent modulators of extracellular matrix synthesis in cell culture and stimulate matrix synthesis in wounds and other remodeling tissues. The role of endogenous TGF-βs in remodeling tissues is less well defined. Vascular remodeling in the pulmonary arteries of patients with primary pulmonary hypertension is characterized, in part, by abnormal deposition of immunohistochemically detectable procollagen, thereby identifying actively remodeling vessels. We used this marker of active matrix synthesis to begin defining the in vivo role of TGF-β in the complex milieu of actively remodeling tissues. Immunohistochemistry using isoform-specific anti-TGF-β antibodies was performed to determine whether TGF-β was present in actively remodeling hypertensive pulmonary arteries 20 to 500 μm in diameter. Intense, cell-associated TGF-β3 immunoreactivity was observed in the media and neointima of these hypertensive muscular arteries. Immunostaining was present, but less intense, in normal arteries of comparable size. TGF-β2 immunoreactivity was observed in normal vessels and was increased slightly in hypertensive vessels, in a pattern resembling TGF-β3 immunoreactivity. No staining was associated with the adventitia. TGF-β1 immunostaining was either faint or absent in both normal and hypertensive vessels. Comparison of procollagen and TGF-β localization demonstrated that TGF-β2 and TGF-β3 co-localized at all sites of procollagen synthesis. However, TGF-β was observed in vessels, or vascular compartments, where there was no procollagen synthesis. Procollagen immunoreactivity was not present in normal vessels that showed immunoreactivity for TGF-β2 and TGF-β3. These observations suggest: a) the stimulation of procollagen synthesis by TGF-β in vivo is more complex than suggested by in vitro studies and b) a potential role for TGF-β2 or TGF-β3, but not TGF-β1, in hypertensive pulmonary vascular remodeling. (Am J Pathol 1994, 144:286–295)***

Transforming growth factor-βs (TGF-βs) are a family of multifunctional molecules that regulate many cellular functions, including cell growth and differentiation.[1,2] 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*.[3]

TGF-βs are also potent modulators of extracellular matrix synthesis. Exogenously administered TGF-β1 induces collagen and other extracellular matrix protein synthesis, such as fibronectin, *in vitro*[4–10] and *in vivo*.[11,12] 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 messenger (m)RNA levels precede the development of pulmonary[13,14] and hepatic fibrosis,[15] TGF-β1 is associated with type I procollagen and fibronectin gene expression in idiopathic pulmonary fibrosis,[16] and neutral-

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izing antibodies that block the action of TGF-β1 inhibit the accumulation of extracellular matrix in an animal model of glomerulonephritis.[17]

It is unclear, however, whether the extracellular matrix synthesis observed in vascular remodeling is induced by TGF-β. Increases in neointimal steady-state levels of fibronectin and procollagen mRNA, following carotid endarterectomy, correlate better with the onset of neointimal formation than with increases in TGF-β1 mRNA.[18] Moreover, TGF-β1 immunoreactivity and tropoelastin gene expression are found in separate, discrete regions of the medial layer of remodeling lobar pulmonary arteries in a hypoxic model of pulmonary hypertension.[19,20] Together, these studies suggest TGF-β1 may not directly participate in modulating extracellular matrix synthesis associated with vascular remodeling. The role of the other two mammalian isoforms of TGF-β, TGF-β2, and TGF-β3 in vascular remodeling is not as well studied, although increases in TGF-β activity precede vascular remodeling in a chronic embolic model of pulmonary hypertension, using an assay that does not distinguish between different isoforms of TGF-β.[21] Whether temporal or spatial correlations exist between TGF-β and extracellular matrix synthesis in human vascular remodeling is even less defined.

Primary pulmonary hypertension is characterized by the abnormal presence of smooth muscle cells in normally nonmuscular compartments or segments of the vessel, and the abnormal deposition of connective tissue proteins in both the large elastic arteries and the small muscular arteries and arterioles.[22,23] Recent studies, using an antibody capable of distinguishing type I procollagen from fully processed, mature collagen[24] provided evidence of persistent collagen synthesis in many, but not all, small muscular pulmonary arteries of patients with severe primary pulmonary hypertension.[25] Furthermore, procollagen was detected in different compartments in different vessels, even within tissue sections from the same patient. This pathological and immunohistological heterogeneity facilitates studying the role of putative mediators of vascular remodeling *in vivo*. For example, the presence of a TGF-β isoform only in vessels actively synthesizing procollagen would strongly suggest its participation in extracellular matrix synthesis *in vivo*. The purpose of this study was to begin defining the role of endogenous TGF-β in remodeling tissues by: 1) determining whether TGF-βs are present within hypertensive pulmonary arteries and 2) defining the spatial relationship between TGF-β protein localization and procollagen synthesis.

## Materials and Methods

### Tissue

Resected lungs were obtained from eight patients undergoing single-lung transplant surgery for unexplained pulmonary hypertension at Washington University Medical Center. Criteria for primary pulmonary hypertension were similar to those of the Patient Registry for the Characterization of Primary Pulmonary Hypertension.[26] Immediately following surgical resection, lungs were processed for histological and immunological studies. The histology of these specimens has been previously described.[25] Briefly, lungs were fixed for 6 hours 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. Normal lungs were obtained from five lung transplant donors and normal areas of three pathological specimens following resection for lung cancer.

Significant tissue degradation in normal donor lungs is unlikely. Acceptable donors are usually in better physiological condition compared to rejected donors, and donor tissues appear normal both grossly and histologically. Furthermore, the pattern of TGF-β immunoreactivity observed in these normal human lungs is not unique. We observe the same patterns of TGF-β immunoreactivity in normal bovine and murine pulmonary arteries fixed immediately after sacrifice.

### Antibodies

TGF-β isoform-specific antibodies were prepared as described.[27] Briefly, peptides of each TGF-β isoform were synthesized, purified, and coupled to keyhole limpet hemocyanin (KLH) for immunization of rabbits. For TGF-β1 and TGF-β2, peptides corresponding to residues 4 to 19 of the mature protein were used as immunogens. For TGF-β3, residues 9 to 20 were used. Each antiserum was purified by ammonium sulfate precipitation followed by peptide affinity chromatography using the respective peptide as immunogen. Each anti-peptide antibody was tested for both immunoreactivity with the corresponding intact TGF-β molecule and for cross-reactivity with each other TGF-β isoform by Western blot analysis. Each anti-peptide antibody to the

three isoforms of TGF-β only reacted with the appropriate and corresponding intact molecule; no cross-reactivity was observed. The specificity of the individual anti-peptide antibody was further demonstrated following complete absorption of the immunoreactivity with 10 mol/L excess of the corresponding peptide. Differences in staining intensities observed for each TGF-β isoform are not due to differences in antibody affinity, because the relative intensities observed on Western blot analysis are equivalent.[27] Moreover, equivalent staining with each anti-TGF-β isoform antibody is observed in other cells and tissues[27] and within alveolar macrophages and bronchoepithelium from normal and hypertensive lungs (see below, Figure 3, C, D and E, demonstrating macrophage immunoreactivity).

M-57 is a rat monoclonal antibody raised against the amino-terminal propeptide of human procollagen type I (gift of Dr. J. McDonald, Mayo Clinic, Scottsdale, AZ) and, therefore, can identify sites of active collagen deposition. Combined immunohistochemistry/*in situ* hybridization on identical tissue sections with this antibody confirmed a high concordance between type I procollagen immunoreactivity and gene expression.[25]

## *Immunohistochemistry*

Sections were prepared for immunoperoxidase staining as described.[25] 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-(Ig) binding sites were blocked with normal goat or horse serum. Sections were subsequently incubated overnight at 4 C with TGF-β isoform-specific antibodies (2.5 μg/ml) or M-57 anti-type I procollagen antibody (1:400). 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 (1:1,600 dilution; Vector Laboratories, Burlingame, CA), washed, and incubated for 60 minutes with horseradish peroxidase-streptavidin (1:400 dilution; Vector). 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*

Immunohistochemistry was performed with peptide affinity-purified, isoform-specific anti-TGF-β peptide antibodies to determine whether TGF-βs were present in hypertensive pulmonary arteries. The pattern of immunohistochemical staining (described below) was similar in all patients regardless of gender, age, or clinical course. Representative hypertensive pulmonary arteries from two separate patients are shown (Figures 1 & 2 and 3 & 4, respectively) following staining for TGF-β1 (panels C), TGF-β2 (panels D), and TGF-β3 (panels E). Results are summarized in Table 1.

A cell-associated pattern of TGF-β2 and TGF-β3 immunoreactivity was present in nearly all hypertensive pulmonary arteries measuring between 20 to 500 μm (Figures 1 to 4, panels D and E), but, uniformly, TGF-β3 immunostaining was more intense than staining for TGF-β2. TGF-β2 and TGF-β3 immunoreactivity co-localized in the neointimal or medial layers. However, the pattern of medial and neointimal staining varied widely, even within the same tissue section. For example, in some vessels, both the medial layer and neointima were immunoreactive, whereas in other vessels of comparable size, only the neointima or medial layer stained for TGF-β2 and TGF-β3. The endothelium of many of the vessels examined were simultaneously immunoreactive for TGF-β2 and TGF-β3 but endothelial immunoreactivity was not observed in all of these vessels. The frequency and intensity of TGF-β1 immunoreactivity in these hypertensive pulmonary arteries (Figures 1 to 4, panels C) was markedly less when compared to TGF-β2 or TGF-β3 immunoreactivity (Figures 1 to 4, panels D and E). TGF-β immunoreactivity for all three isoforms was not observed in the adventitia. All three isoforms were present in most alveolar macrophages and bronchoepithelium (data not shown).

Immunohistochemistry for procollagen and each TGF-β isoform were compared on serial sections to determine whether procollagen co-localized with TGF-β2 and TGF-β3. TGF-β2 and TGF-β3 immunoreactivity was always observed wherever procollagen staining was present (Figures 1, 2, and 3, panels B, D, and E), although TGF-β2 and TGF-β3 immunoreactivity was also observed in areas devoid of procollagen staining. For example, less than 5% of all hypertensive vessels did not stain for procollagen, indicating they were not remodeling, despite the presence of immunoreactive TGF-β2 and TGF-β3 within the medial layer or neointima (Figure 4, B, D, and E). Most frequently, however, TGF-β2

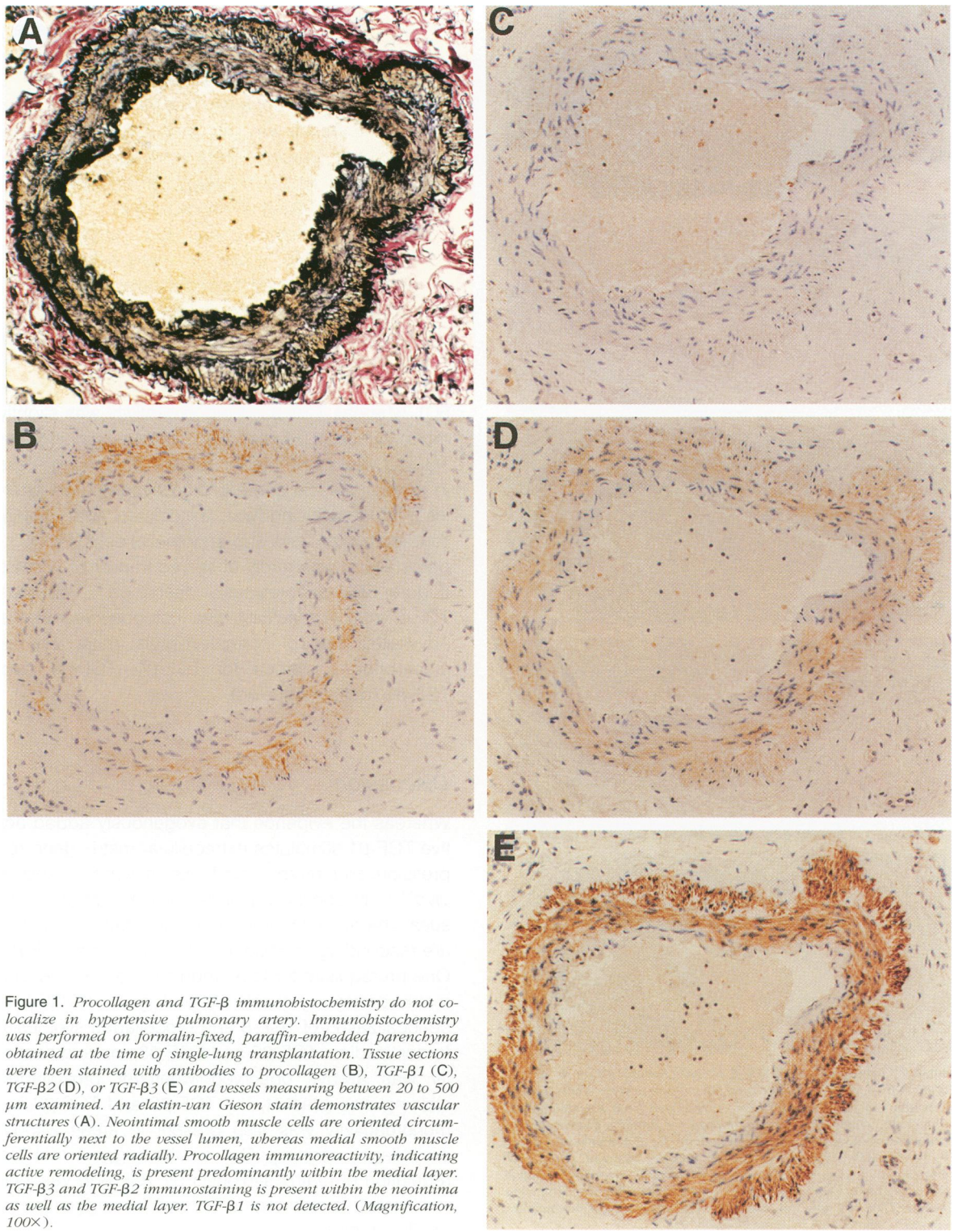


Figure 1. *Procollagen and TGF-β immunohistochemistry do not co-localize in hypertensive pulmonary artery. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded parenchyma obtained at the time of single-lung transplantation. Tissue sections were then stained with antibodies to procollagen* (B), *TGF-β1* (C), *TGF-β2* (D), *or TGF-β3* (E) *and vessels measuring between 20 to 500 μm examined. An elastin-van Gieson stain demonstrates vascular structures* (A). *Neointimal smooth muscle cells are oriented circumferentially next to the vessel lumen, whereas medial smooth muscle cells are oriented radially. Procollagen immunoreactivity, indicating active remodeling, is present predominantly within the medial layer. TGF-β3 and TGF-β2 immunostaining is present within the neointima as well as the medial layer. TGF-β1 is not detected. (Magnification, 100×).*

and TGF-β3 immunoreactivity was observed in a layer of the arterial wall that did not stain for procollagen, despite co-localization in other layers of the vessel. For example, procollagen staining was often observed in the neointima only (Figures 2B and 3B), although TGF-β2 and TGF-β3 immunoreactivity was observed in both neointima and media (Figures 2 and 3, panels D and E). Less frequently, procolla-

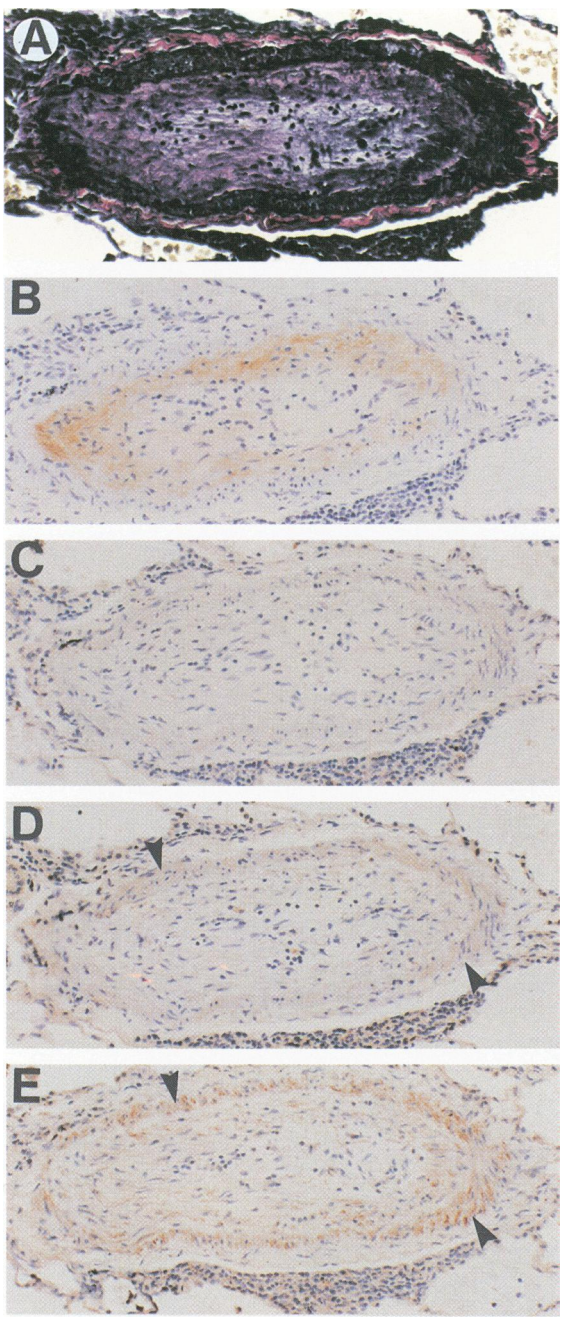


Figure 2. *Procollagen and TGF-β immunohistochemistry do not co-localize in occluded hypertensive pulmonary artery. Immunohistochemistry was performed on lung parenchyma from a second patient undergoing single-lung transplantation for primary pulmonary hypertension. Procollagen immunoreactivity* (B) *is present only within the neointima of this occluded artery. TGF-β3* (E) *and TGF-β2* (D) *immunoreactivity is most intense in the medial layer* (*arrowhead*), *although faint staining for TGF-β3 is observed within the neointima. TGF-β1* (C) *is not detected. An elastin-van Gieson stain demonstrates vascular structures* (A). (*Magnification, 100×*).

gen was present within the medial layer (Figure 1B) despite the presence of TGF-β2 and TGF-β3 immunoreactivity in both the neointima and medial layer (Figure 1, D and E). In summary, TGF-β and procollagen immunoreactivities did not precisely co-localize in remodeling hypertensive pulmonary arteries. Furthermore, there was no specific correlation detected between vessel histology and TGF-β immunoreactivity. Because vessel histology also does not correlate well with procollagen immunoreactivity,[25] this suggests there is no specific relationship between vessel histology, procollagen immunoreactivity, or TGF-β immunoreactivity.

Immunohistochemistry was also performed with normal pulmonary tissue to determine whether TGF-βs were present in normal, nonremodeling vessels. In general, normal pulmonary arteries measuring between 20 to 500 μm displayed a pattern of immunostaining similar to hypertensive vessels (Figure 5). TGF-β1 immunoreactivity was faint (Figure 5C), whereas TGF-β2 immunoreactivity was slightly more intense (Figure 5D). Immunostaining for TGF-β3 was most intense (Figure 5E), though less in normal vessels compared to hypertensive arteries. Immunostaining was associated with medial smooth muscle cells and endothelial cells. Procollagen staining was rarely observed in normal vessels (Figure 5B), as previously described.[25] Whereas TGF-β immunoreactivity was not observed in the adventitial layer of normal vessels, most alveolar macrophages stained for TGF-β1, TGF-β2, and TGF-β3 (data not shown).

## Discussion

Whereas the evidence that exogenously added active TGF-β1 stimulates extracellular matrix gene expression and protein synthesis *in vitro*[4–10] and *in vivo*[11,12] is convincing, it is not clear whether tissues characterized by increased matrix synthesis are responding to endogenously produced TGF-βs. One prerequisite for local regulation of extracellular matrix protein synthesis by endogenous TGF-β is co-localization of TGF-β and procollagen synthesis. In our studies, TGF-β2 and TGF-β3 immunoreactivity was always observed wherever procollagen staining was present, but TGF-β2 and TGF-β3 immunoreactivity was also present in areas without procollagen staining. Our results suggest that the regulation of matrix synthesis by TGF-β in vascular remodeling is more complicated than suggested by *in vitro* studies.

There are several possibilities to explain why TGF-β is present in vessels but not always associated with procollagen staining. Once synthesized, TGF-β is stored intracellularly until secreted as an inactive peptide complex.[1] After secretion, TGF-β

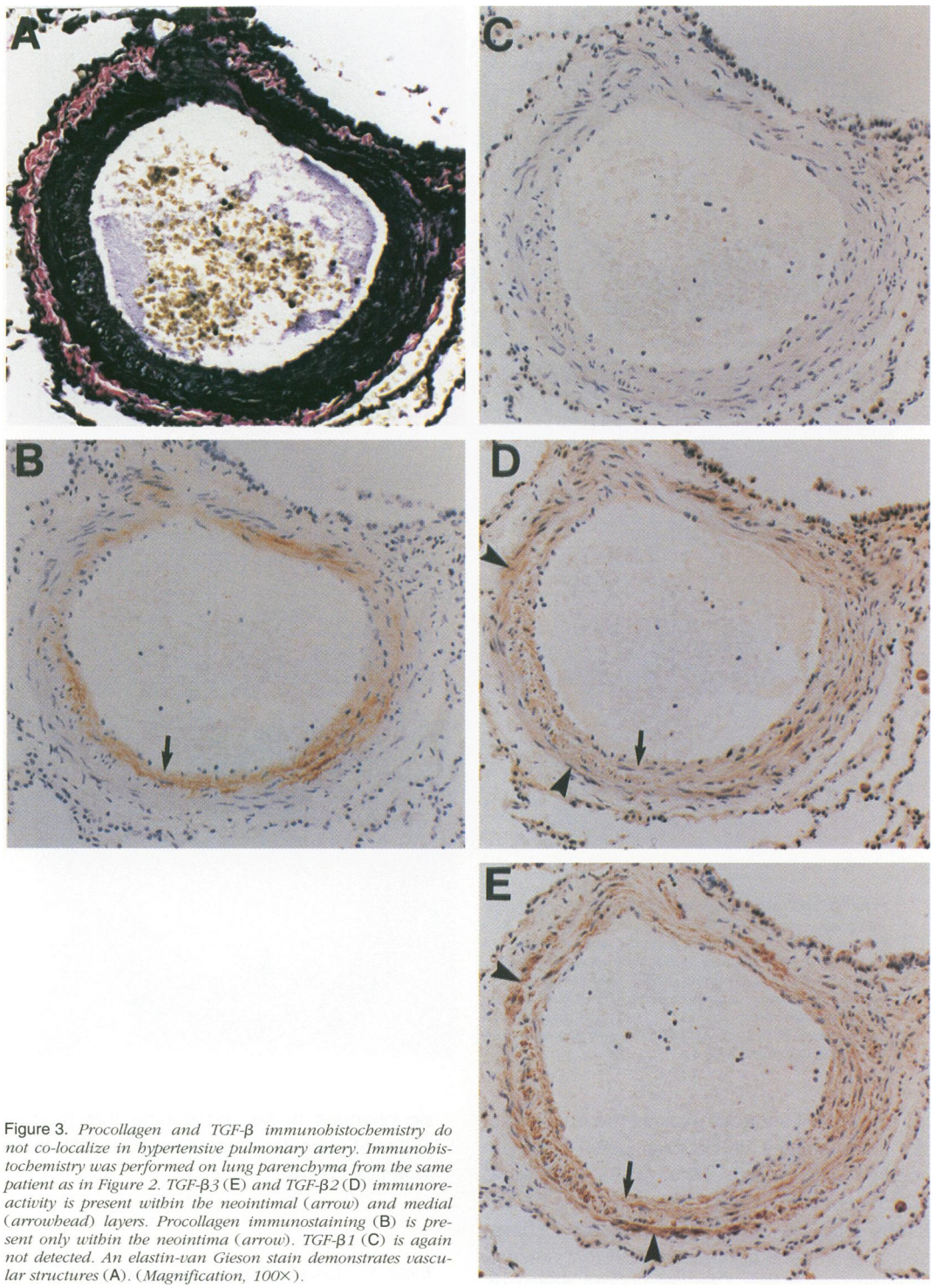


Figure 3. *Procollagen and TGF-β immunohistochemistry do not co-localize in hypertensive pulmonary artery. Immunohistochemistry was performed on lung parenchyma from the same patient as in Figure 2. TGF-β3* (E) *and TGF-β2* (D) *immunoreactivity is present within the neointimal (arrow) and medial (arrowhead) layers. Procollagen immunostaining* (B) *is present only within the neointima (arrow). TGF-β1* (C) *is again not detected. An elastin-van Gieson stain demonstrates vascular structures* (A). *(Magnification, 100×).*

must be activated, although the activation mechanism is poorly understood. Active TGF-β can bind extracellular matrix proteins, such as thrombospondin,[28] type IV collagen,[29] and decorin,[30] that may maintain the growth factor in an inactive state.[30] TGF-β may also bind cell-associated proteins, such as betaglycan, which do not function in signal transduction.[1] In these hypertensive pulmonary arteries, TGF-β immunoreactivity seemed cell-associated rather than associated with the extracellular matrix. It is unclear whether the antibody is identifying latent, intracellular TGF-β or TGF-β associated with

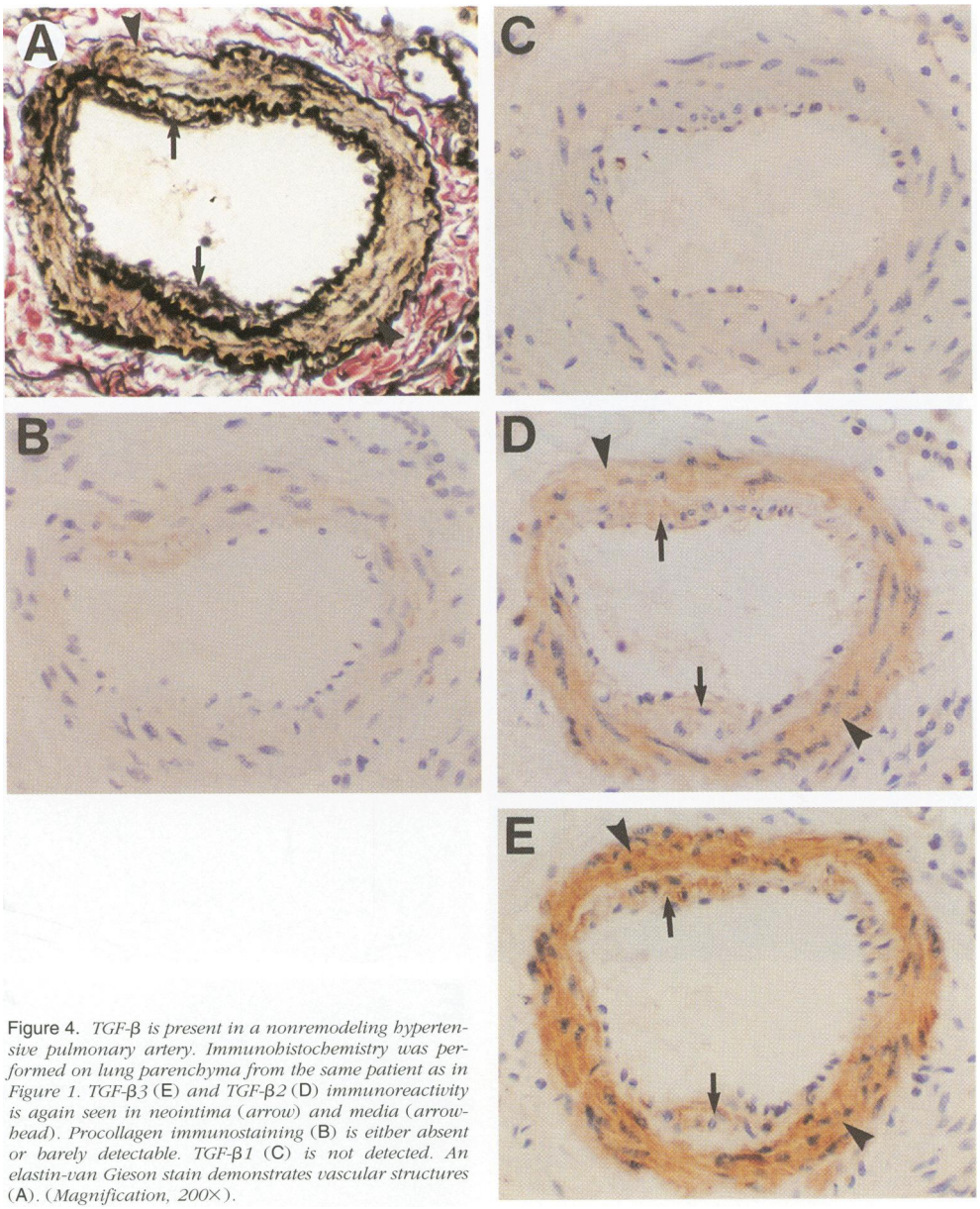


Figure 4. *TGF-β is present in a nonremodeling hypertensive pulmonary artery. Immunohistochemistry was performed on lung parenchyma from the same patient as in Figure 1. TGF-β3* (E) *and TGF-β2* (D) *immunoreactivity is again seen in neointima (arrow) and media (arrowhead). Procollagen immunostaining* (B) *is either absent or barely detectable. TGF-β1* (C) *is not detected. An elastin-van Gieson stain demonstrates vascular structures* (A). *(Magnification, 200×).*

proteins on the cell surface because the antibodies used for these studies do not distinguish between the precursor and mature forms of TGF-β. Therefore, we cannot exclude the possibility that active TGF-β molecules may be present in areas of active remodeling and latent, or inactive, TGF-β is present in nonremodeling areas. Conversely, the ability of vascular smooth muscle cells to respond to TGF-β *in vivo* may vary, so that smooth muscle cells in nonremodeling vessels are unable to synthesize procollagen despite the presence of active TGF-β. This may result from the need for additional autocrine or paracrine permissive factors, alterations in signal-transducing TGF-β receptors, or other intrinsic changes in cell phenotype that modulate the ability of vascular smooth muscle cells to respond to active TGF-β.

Table 1. *Vascular TGF-β Immunoreactivity in Normal and Hypertensive Pulmonary Arteries*

| Isoform | Normal | Hypertensive |
|---|---|---|
| TGF-β1 | +/– | – |
| TGF-β2 | +/++ | ++ |
| TGF-β3 | ++ | +++ |

The number of + signs indicates relative intensity of TGF-β isoform-specific immunostaining. A – sign indicates staining was not observed.

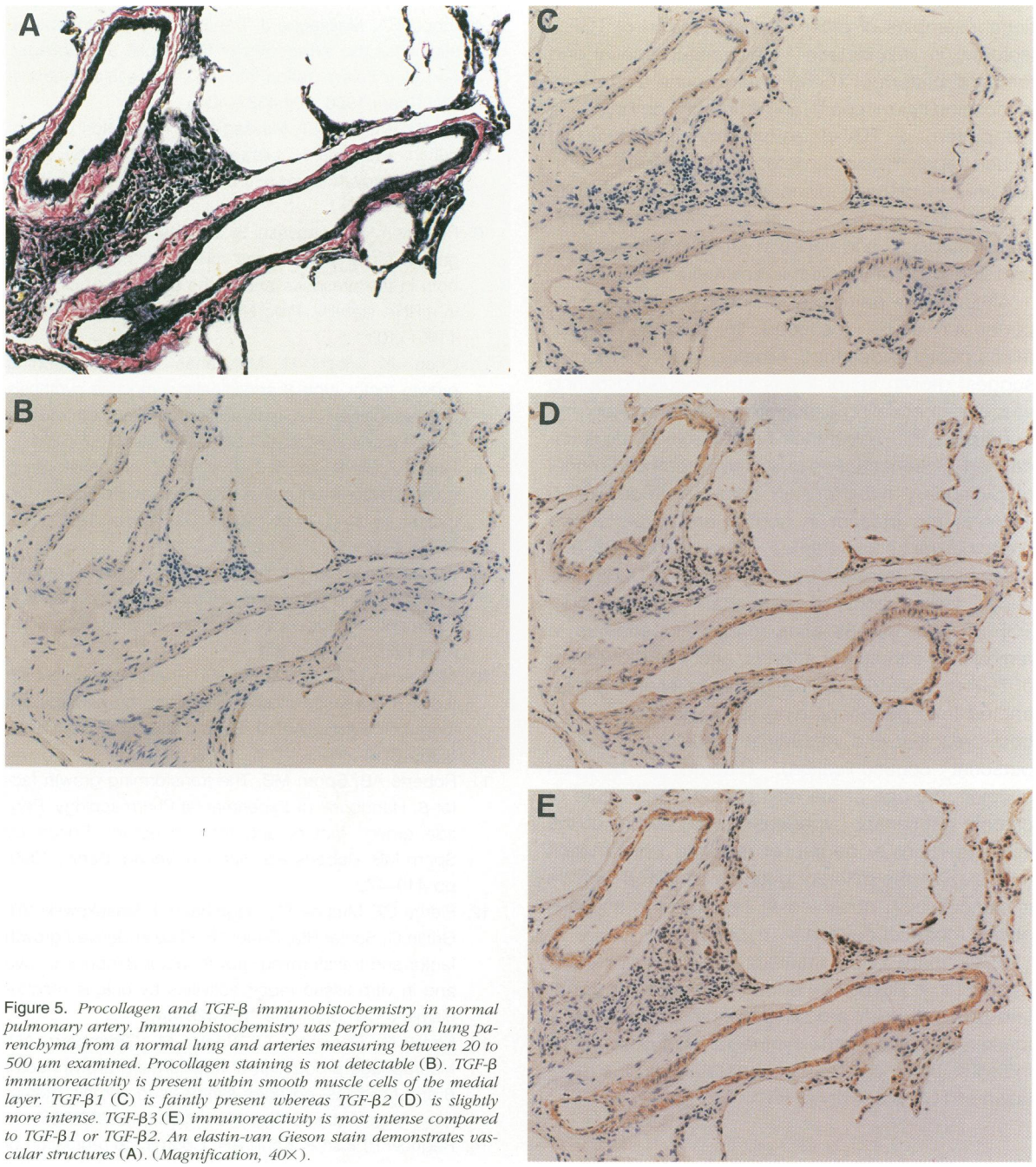


Figure 5. *Procollagen and TGF-β immunohistochemistry in normal pulmonary artery. Immunohistochemistry was performed on lung parenchyma from a normal lung and arteries measuring between 20 to 500 μm examined. Procollagen staining is not detectable* (B). *TGF-β immunoreactivity is present within smooth muscle cells of the medial layer. TGF-β1* (C) *is faintly present whereas TGF-β2* (D) *is slightly more intense. TGF-β3* (E) *immunoreactivity is most intense compared to TGF-β1 or TGF-β2. An elastin-van Gieson stain demonstrates vascular structures* (A). *(Magnification, 40×).*

Although each isoform of TGF-β shares significant homology with the others, each isoform has unique promoters.[31–33] TGF-β3 immunoreactivity in normal and hypertensive pulmonary arteries appeared most intense compared to TGF-β1 and TGF-β2, and TGF-β3 isoform staining appeared increased in hypertensive vessels compared to normal vessels. TGF-β2 immunoreactivity was only slightly more intense in hypertensive arteries compared to normal vessels, and faint TGF-β1 immunoreactivity appeared only infrequently in both normal and hypertensive pulmonary arteries. The factors that are responsible for the marked increase in TGF-β3, but not TGF-β1, in hypertensive vessels are unknown. Nevertheless, emerging evidence suggests that, despite similar biological activities of these isoforms *in vitro*, specific biological effects *in vivo* may result from unique expression and localization of a particular isoform.[3] Our observation that TGF-β3, and to a lesser extent TGF-β2, immunoreactivity appears increased in hypertensive vessels compared to TGF-β1 is consistent with that hypothesis. Addi-

tional examples of differential expression of TGF-β isoforms *in vivo* include 1) the unique spatial and temporal pattern of TGF-β isoform gene expression in developing murine[34] and human[35] embryos; 2) the pattern of TGF-β isoform immunostaining in wound repair[36]; and 3) detection of specific TGF-β isoforms in different disease states. For example, TGF-β2, but not TGF-β1, is associated with intraocular fibrosis[37] and vascular procollagen gene expression in dermal systemic sclerosis.[38]

Although the pathology of primary pulmonary hypertension is well described, the pathogenesis remains poorly understood. Studies in animal models suggest growth factor levels are modulated during vascular remodeling associated with pulmonary hypertension,[19,21,39] but their precise role remains undefined. Recent studies in a chronic embolic model of pulmonary hypertension show all three TGF-β isoforms are present in normal and hypertensive preacinar muscular arteries, although no TGF-β is detectable in smaller intraacinar arteries. During the early development of pulmonary hypertension, TGF-β1 and TGF-β3 immunoreactivity appears in remodeling intraacinar arteries, and the abundance of TGF-β1 and TGF-β3 mRNA increases in RNA extracted from peripheral lung tissue (which includes both preacinar and intraacinar vessels) (E. Perkett, personal communication). Differences between species, the age of the animal or patient, acute or chronic pulmonary hypertension, the nature of the initiating injury or degree of resulting inflammation may explain the different patterns of TGF-β isoform immunostaining observed in our patients and this animal model of pulmonary hypertension.

In summary, TGF-β immunoreactivity is present in hypertensive pulmonary arteries but does not correlate precisely with procollagen immunoreactivity, a marker of active matrix synthesis. This observation indicates the importance of understanding the regulation of TGF-β activity *in vivo*.

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