Regression of Hypertrophied Rat Pulmonary Arteries in Organ Culture Is Associated With Suppression of Proteolytic Activity, Inhibition of Tenascin-C, and Smooth Muscle Cell Apoptosis

Kyle Northcote Cowan, Peter Lloyd Jones, Marlene Rabinovitch

Abstract—Increased elastase activity and deposition of the matrix glycoprotein tenascin-C (TN), codistributing with proliferating smooth muscle cells (SMCs), are features of pulmonary vascular disease. In pulmonary artery (PA) SMC cultures, TN is regulated by matrix metalloproteinases (MMPs) and mechanical stress. On attached collagen gels, MMPs upregulate TN, leading to SMC proliferation, whereas on floating collagen, reduced MMPs suppress TN and induce SMC apoptosis. We now investigate the response of SMCs in the whole vessel by comparing attached and floating conditions using either normal PAs derived from juvenile pigs or normal or hypertrophied rat PAs that were embedded in collagen gels for 8 days. Normal porcine PAs in attached collagen gels were characterized by increasing activity of MMP-2 and MMP-9 assessed by zymography and TN deposition detected by Western immunoblotting and densitometric analysis of immunoreactivity. PAs on floating collagen showed reduced activity of both MMPs and deposition of TN. Tenascin-rich foci were associated with proliferating cell nuclear antigen immunoreactivity, and TN-poor areas with apoptosis, by terminal deoxynucleotidyl transferase–mediated nick end labeling assay, but no difference in wall thickness was observed. Although normal rat PAs were similar to piglet vessels, hypertrophied rat PAs showed an amplified response. Increased elastase, MMP-2, TN, and elastin deposition, as well as SMC proliferating cell nuclear antigen positivity, correlated with progressive medial thickening on attached collagen, whereas reduced MMP-2, elastase, TN, and induction of SMC apoptosis accompanied regression of the thickened media on floating collagen. In showing that hypertrophied SMCs in the intact vessel can be made to apoptose and that resorption of extracellular matrix can be achieved by inhibition of elastase and MMPs, our study suggests novel strategies to reverse vascular disease. (Circ Res. 1999;84:1223-1233.)

Key Words: extracellular matrix • tenascin-C • pulmonary vascular regression • apoptosis • proteinase

Pulmonary hypertension can be a rapidly progressive and fatal disease characterized by changes in vascular structure and function associated with endothelial cell injury,1 neomuscularization of peripheral arteries, and smooth muscle cell (SMC) proliferation and migration into the neointima.2 This is accompanied by remodeling of the extracellular matrix (ECM) by proteinases3,4 and by increased expression and deposition of the ECM components elastin, collagen,5,6 fibronectin,7,8 and tenascin-C (TN).8,9

Our laboratory has studied the pathophysiologic role of TN in pulmonary vascular disease (PVD). In lung biopsies from patients with pulmonary hypertension, expression of TN, observed in the hypertrophied media and neointima, was associated with proliferating cell nuclear antigen (PCNA)–positive cells.8 A cause-and-effect relationship between TN and SMC proliferation was shown in cell culture studies in which TN amplified the proliferative response to growth factors.9 The mechanism, related to alterations in SMC shape, was explored by comparing cells on attached or floating collagen gels.10 In intact collagen gels, matrix metalloproteinase (MMP)–2 activity and ligation of β integrins by cryptic RGD (arginine-glycine-aspartic acid) sites in proteolyzed collagen led to TN production. Clustering of α,β integrins, by newly synthesized TN, was associated with focal adhesion contact formation in which epidermal growth factor receptors were accumulated and, on ligation, efficient receptor tyrosine phosphorylation and nuclear growth signals were evident. Alternatively, on floating collagen, MMP-2 activity declines, endogenous TN levels fall, and SMC apoptosis is evident.10 These studies raise the question whether SMCs in their native vascular environment might also respond to physical changes by altering MMP and TN production in concert with proliferation or apoptosis. To investigate this, we cultured intact porcine pulmonary arteries (PAs) on attached and on
floating collagen gels. On attached collagen, we documented increased MMP-2 and MMP-9 activity and deposition of TN. By contrast, in floating cultures, suppression of TN and a relative reduction in MMP activity were observed. The presence of both TN-rich and TN-poor foci correlated with proliferation and apoptosis, respectively, but these effects did not appear to significantly impact on wall thickness in normal vessels.

We reasoned, however, that hypertensive vessels, already in a state of active remodeling, may be more responsive to manipulation of TN. In addition, an amplified deposition of TN may occur through heightened activation of MMPs, ECM proteolysis by the increased serine elastase activity in hypertensive PAs. This, in turn, may augment the SMC proliferative response, whereas withdrawal of TN might mediate a more profound apoptotic response.

To investigate this, we cultured hypertrophied rat PAs on attached or floating collagen gels and observed continued, progressive medial thickening on attached gels, whereas vessels placed on floated gels underwent regression to control (saline) levels. This was associated with a suppression in elastase and MMP activity (primarily MMP-2) and loss of TN. Moreover, regression of hypertrophy correlated with reduced elastin, indicating that a coordinated loss of both vascular cellularity and ECM components was achieved.

**Materials and Methods**

**Preparation of Explants**
PAs were collected and prepared for organ culture as previously described. Freshly harvested porcine PAs obtained from the local slaughterhouse were transported to the laboratory in sterile PBS and 2% antibiotics/antimycotics (GIBCO). While the endothelial surface was kept moist and protected, the periadventitial fat was removed and PAs were cut along their long axes. Rectangular pieces of full-thickness vessel wall (4 × 10 mm) were cut and placed in culture.

In the second series of experiments, PAs were harvested from adult male Sprague-Dawley rats (300 g) (Charles River Laboratories, Saint-Laurent, Quebec, Canada) 21 days after a subcutaneous injection of the alkaloid toxin monocrotaline (MCT) (60 mg/kg) or saline (controls). MCT-induced PVD is well established, and at 21 days after injection of the toxin up to a doubling of the wall thickness of the PAs, 3 equidistant measurements were recorded, and in rat PAs, 25 measurements in consecutive fields of view (290-μm2 fields at an original magnification of ×400) were recorded and means calculated. Elastic laminae were assessed by counting the number of continuous elastic laminae per PA. The total amount of elastin was assessed by planimterization of both elastic laminae and interlamellar fragments of elastin. These assessments were performed in 5 random fields per vessel at the above magnification.

**Assessment of Elastolytic Activity**
Conditioned media (380 μL) were incubated at 37°C with 20 μL (200 μg) [3H]elastin produced by radiolabeling purified insoluble elastin from bovine nuchal ligament (Elastin Products Co) with [3H]NaBH₄ (New England Nuclear) as previously described. After 24 hours, the radioactivity in 250 μL of the supernatant was determined by liquid scintillation spectrometry as a measure of elastolysis. To control for nonenzymatic degradation, radiolabeled elastin was incubated with medium from tissue-free cultures. Elastase activity was related to a standard curve generated with human leukocyte elastase (0.075 to 5.0 ng) (Elastin Products Co).

**Western Immunoblotting**
MMP-2, MMP-9, and TIMP-1 Western immunoblotting was performed on 12% polyacrylamide gels using anti-human monoclonal antibodies (1:50) (Oncogene Science) for porcine extracts and an anti-MMP-2 rabbit polyclonal antiserum for rat extracts (a kind gift from Drs M. Silverman and M. Ailenburg [Department of Medicine, University of Toronto]). An anti-human cellular TN polyclonal antibody (1:50) (Sigma) for porcine tissue and an anti-rat polyclonal antibody raised against formaldehyde-fixed TN (1:100) (a generous gift from Dr Harold Erickson [Duke University Medical Center, Durham, NC]) was used to detect TN separated on 6% gels. Visualization of immunoreactive bands was achieved with a horse-radish peroxidase–conjugated secondary antibody (GIBCO) followed by enhanced chemiluminescence (Amersham) and normalized by comparison with a Coomassie blue–stained gel.

**Northern Blotting**
From culture, PAs were immediately placed in 5 mL of TRIzol, total RNA was extracted according to that protocol (GIBCO-BRL), and Northern blotting was performed as previously described. A 250-bp cDNA probe derived from the seventh fibronectin type III constant domain of rat TN was used on 8-μg samples. The relative quantity of TN mRNA in each sample was analyzed by densitometry and corrected for loading conditions by direct comparison with ethidium bromide–stained 28S rRNA and by hybridization with a 600-bp cDNA probe for rat GAPDH.

**Immunohistochemistry**
Samples were removed from the collagen gels and fixed in 2% paraformaldehyde. They were then embedded in paraffin and cut into 5-μm-thick sections. Immunohistochemistry for TN was performed with the species-specific TN primary antibodies described above (same dilutions) in an overnight incubation at 4°C. Binding was visualized using the Vectastain ABC System (Vector Laboratories). Control sections were treated with normal rabbit isotypic IgG (DAKO). Nuclei were counterstained using hematoxylin.
The relative abundance of TN was graded quantitatively in 5 random fields (290-μm² fields at an original magnification of ×400) per sample using the Image-Pro Plus program for a Macintosh computer (Media Cybernetics). Planimetry and densitometry were performed on positive staining above the designated “background.” Multiplication of the total positive area and the average density provided a relative densitometric measurement of TN deposition for each field, and means were calculated.

PCNA was detected after nuclease digestion and incubation with an anti-PCNA monoclonal primary antibody (1:100) (DAKO). Control sections were incubated with normal mouse isotopic IgG (DAKO), and a normal human skin section was used as a positive control. Antibody binding was visualized using 3,3′-diaminobenzidine (Sigma) and a substrate intensifier (Amersham). Cytoplasmic counterstaining was performed using cosin.

Apoptosis was detected by an in situ detection system following the manufacturer’s protocol (Apoptag, Oncor). On porcine sections, the secondary antibody was peroxidase-conjugated, whereas we used a fluorescein-conjugated secondary antibody on rat sections. A normal skin sample was used as a positive control, and sections incubated in the absence of either antibody or enzyme were used as negative controls. Nuclear counterstaining was performed on porcine sections with methylene green and on rat sections with propidium iodide.

The presence of proliferating and apoptotic cells was also quantitatively assessed in 10 randomly selected fields (290-μm² fields at an original magnification of ×400). The number of positive cells and the total number of cells (nuclei identified by propidium iodide counterstaining) per field were counted, a percentage of positive cells was calculated, and a mean percentage was generated.

Detection of Necrosis
Cellular necrosis was assessed with a lactate dehydrogenase release assay (Sigma) performed on 120 μL of conditioned medium and according to the manufacturer’s specifications. Background, determined using both serum-free medium and medium containing 5% FBS cultured in the absence of a tissue section, was subtracted. Assay sensitivity was determined using supernatants from PA SMCs after freeze-thaw cycles.

Statistical Analysis
Experiments were performed at least 3 times (exact numbers are indicated in figure legends), and values are expressed as mean±SEM. Statistical significance was determined using 1-way ANOVA followed by the Fisher least significant difference test of multiple comparisons to establish differences between individual groups.

Results
MMP Activity in Porcine PAs
Because MMP-2 regulates TN deposition in cultured PA SMCs,16 net MMP activity was assessed in PA tissue extracts by gelatin zymography and reverse zymography. Gelatinolytic bands at ≈83 kDa and a triplet at ≈52, 56, and 60 kDa were detected (Figure 1) and confirmed to result from MMP activity through their abrogation after incubation in EDTA (data not shown). Furthermore, some of these bands were characterized by native Western immunoblotting to be MMP-9 (83 kDa) and the active and latent (52- and 56-kDa) forms of MMP-2, respectively (data not shown). The enzyme responsible for the 60-kDa gelatinolytic band was not identified. Qualitative analysis of the zymograms showed a reproducible increase in MMP-2 activity in attached cultures on day 5 compared with day 0. This increase in MMP-2 was evident in both attached and floating cultures by day 8; however, in attached cultures, but not in floating cultures, an

![Figure 1. Representative gelatin zymogram of porcine PA organ culture tissue collected from attached and floating cultures at 0, 1, 3, 5, and 8 days is shown. Bands at ≈83, 56, and 52 kDa reflecting MMP-9 and MMP-2 (active and latent form), respectively, are evident in both attached (Att) and floating (Fl) conditions. S indicates molecular weight standards.](Image 321x126 to 523x332)

![Figure 2. A, Representative Western immunoblot for TN in porcine PA organ culture tissue collected from attached and floating cultures at 0, 1, 3, 5, and 8 days. B, Densitometric analyses from 3 different experiments. TN was identified in these tissues as a ∼220-kDa immunoreactive band that was increased in attached cultures and, in contrast, was reduced in floating cultures. Data are mean±SEM of 3 vessels at each time point. *P<0.05 comparing attached and floating cultures; †P<0.05 compared with day 0.)](Image 334x589 to 510x718)
Figure 3. Photomicrographs of porcine PA organ culture tissue sections, although taken from an attached culture, are representative of samples collected from attached and floating cultures at 8 days and immunostained for TN (A and B) and PCNA (C) and after TUNEL assay (D). Magnification: A, ×100; B, C, and D, ×400. In panels B, C, and D, the boundary between a TN-rich and TN-poor region is illustrated on serially stained sections. C and D show that PCNA positivity (C) codistributes with the TN-rich area and apoptotic cells (D) with the TN-poor area. Bars = 25 μm. Planimetric and densitometric grading of TN from porcine attached and floating cultures from days 0 to 8 is shown in panel E, and counts of proliferating or apoptotic cells calculated from numerous TN-rich and TN-poor regions (see Materials and Methods) are illustrated in panel F. Data are mean ± SEM of 3 vessels at each time point (n = 8 for panel F). *P < 0.05 comparing attached and floating cultures; †P < 0.05 compared with day 0.
culture conditions affect temporal and spatial deposition of TN. On Western immunoblot, TN was identified as a ~220-kDa immunoreactive band that progressively increased in tissue cultured on attached collagen (Figure 2). On attached collagen gels, these vessels continue to thicken over the 8 days (C and E). Vessels on floating gels, however, show a progressive regression of medial thickness over the 8 days (D and E). Bar=25 μm. Measurements of medial hypertrophy (E) showed significant corresponding differences and are associated with modulation of matrix, represented as increases in elastin in attached cultures and decreases in floating cultures. This is assessed by the number of elastic laminae (F) and by relative densitometric units of elastin in the vessel wall (data not shown). Data are mean±SEM of 3 vessels at each time point (n=8 for graph of medial thickness). *P<0.05 comparing attached and floating conditions; †P<0.05 compared with day 0.

Figure 4. Representative photomicrographs of Movat-stained rat PA organ culture tissue collected from attached and floating cultures at 0 and 8 days. Rat PAs taken at 21 days, the point at which they would be placed into culture, show medial hypertrophy (A), in contrast to PAs from saline-injected rats (B). On attached collagen gels, these vessels continue to thicken over the 8 days (C and E). Vessels on floating gels, however, show a progressive regression of medial thickness over the 8 days (D and E). Bar=25 μm. Measurements of medial hypertrophy (E) showed significant corresponding differences and are associated with modulation of matrix, represented as increases in elastin in attached cultures and decreases in floating cultures. This is assessed by the number of elastic laminae (F) and by relative densitometric units of elastin in the vessel wall (data not shown). Data are mean±SEM of 3 vessels at each time point (n=8 for graph of medial thickness). *P<0.05 comparing attached and floating conditions; †P<0.05 compared with day 0.

developed a semiquantitative method to evaluate the sections to take this into account. Planimeterization and densitometry of TN-immunopositive regions revealed an increase in TN on attached collagen gels that was significant by day 5 (P<0.05) (Figure 3E), whereas PAs on floating collagen displayed low levels of immunostaining. The decrease in TN immunostaining preceded the decrease in TN on Western immunoblot, which suggests either a difference in the sensitivity of the methods or an initial conformational change in TN in PAs on floating collagen gels, making it less immunodense on tissue sections.
Tenascin, Proliferating Cells, and Apoptosis

To assess whether TN deposition correlated with proliferating cells, immunostaining for PCNA was used, whereas apoptosis was detected by in situ terminal deoxynucleotidyl transferase–mediated deoxyuridine nick end labeling (TUNEL) assays. TN, accumulating in foci (Figure 3A and 3B), colocalized directly with PCNA-positive cells (Figure 3C), and inversely with apoptotic cells (Figure 3D). Planimetry and densitometry of TN-rich and TN-poor foci further confirmed this association, as regions with high TN deposition had a similarly high proliferation index and low levels of apoptosis, whereas areas with reduced TN exhibited almost an absence of proliferation and a very high percentage of apoptotic cells (Figure 3F). Despite these correlations, changes in wall thickness were not observed when attached and floating vessels were compared (data not shown). If we divide TN staining into negative, minimal, moderate, and intense classifications as judged by relative densitometric units (<0.01, 0.01 to 1, >1 to 10, and >10, respectively [Figure 3F]), it is conceivable that floating cultures were composed of negative and moderate patches of TN and attached cultures were composed of minimal and intense patches. Thus, in both cultures, proliferating and apoptotic cells were balanced (supported by total counts of PCNA-positive and apoptotic cells; data not shown).

Progression and Regression of Hypertrophied Rat PAs

Recent studies suggest that actively remodeling vessels are uniquely dependent on survival signals provided through interaction with the ECM. Consequently, we investigated whether hypertensive vessels, already in a state of active remodeling, may be more responsive to manipulation of TN by attached versus floating conditions. After MCT treatment, a 30% increase in medial hypertrophy was observed when compared with saline controls ($P<0.05$) (Figure 4A, 4B, and 4E). This hypertrophy was not only perpetuated on attached collagen gels, but there was a further 25% increase by day 8 ($P<0.05$) (Figure 4C and 4E), whereas a progressive regression of medial hypertrophy over this time frame was observed on floating collagen (40% decrease over day 0) ($P<0.05$) (Figure 4D and 4E). The progression and regression were not recapitulated by rat saline control vessels cultured in a similar fashion (data not shown) and indicated that normotensive rat PAs behave like the porcine PAs described previously in detail.

Progressive hypertrophy on attached gels was associated with a trend toward an increase in the number of elastic laminae (Figure 4A, 4C, and 4F) and deposition of elastin (data not shown). Regression of medial hypertrophy in floating cultures was associated with resorption of elastin (Figure 4A and 4D) to values comparable with those in saline control vessels in terms of number of laminae (Figure 4F) and elastin densitometry (data not shown).

Elastase and MMP-2 Activity

Because serine elastases are increased in hypertensive PAs, they may play a direct role in the upregulation of TN through the activation of MMPs. Thus, elastolytic activity was examined by elastase assays comparing attached and floating cultures after 8 days in organ culture and showed reduced elastolytic activity in floating vs attached conditions (A). A decrease in proteolytic activity in floating cultures was also observed after Western immunoblotting for MMP-2 (not shown) and gelatin zymography (B). Densitometric quantification of differences in the active form of MMP-2 are shown in panel B; the amount of latent enzyme is similar in tissues on attached and floating gels. Data are mean±SEM of 3 vessels. *$P<0.05$ compared with attached conditions.

Figure 5. Elastase assays were performed on MCT-injected rat PAs after 8 days in organ culture and showed reduced elastolytic activity in floating vs attached conditions (A). A decrease in proteolytic activity in floating cultures was also observed after Western immunoblotting for MMP-2 (not shown) and gelatin zymography (B). Densitometric quantification of differences in the active form of MMP-2 are shown in panel B; the amount of latent enzyme is similar in tissues on attached and floating gels. Data are mean±SEM of 3 vessels. *$P<0.05$ compared with attached conditions.

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latent MMP-2 was detected by zymography in conditioned medium from attached and floating cultures and in similar amounts. The active form of MMP-2 was not observed (data not shown).

Deposition of Tenascin
Ten deposition, assessed by immunohistochemistry, was largely negative in PAs from saline-injected rats (Figure 6B). TN accumulated after MCT injection and was deposited throughout the vessel wall in a cell-associated fashion (Figure 6A). TN deposition is further enhanced on attached collagen (Figure 6C), in contrast to floating cultures in which TN is suppressed (Figure 6D). The marked reduction of TN in floating cultures was consistent with Northern and Western immunoblotting for TN (Figure 6E and 6F). There was a >50% decrease in total TN mRNA, identified as ~7.3- and 6.4-kb alternatively spliced isoforms, with a loss of TN-immunoreactive bands (~230, 220, and 180 kDa) in 3 independent experiments. Extrusion of TN from these cultures, examined by immunoblotting conditioned medium, was not evident.

Figure 6. Representative photomicrographs of TN immunohistochemistry in rat PA tissue reveal an increase in TN deposition 21 days after MCT injection (A) relative to saline-injected controls (B). PA tissue harvested after 8 days in organ culture indicate an accumulation of TN in cultures on attached gels (C), whereas floating PAs reduce TN deposition such that it is nearly absent at day 8 (D). Bar=25 μm. A representative TN Northern blot shows a decrease of total TN mRNA (~7.3- and 6.4-kb alternatively spliced transcripts observed) in tissue on floating compared with attached collagen gels, as compared with 28S rRNA loading control (E). A marked reduction in TN protein (~230-, 220-, and 180-kDa immunoreactive bands) was observed on Western immunoblots (F). Data are mean±SEM of 3 vessels. *P<0.05 compared with attached conditions.
When the vessels are maintained on attached collagen gels, the SMCs respond to mechanical changes in collagen gels. In this study, we have shown that SMCs in the intact vessel, stimulated by mechanical stress or deformation, upregulate TN in the presence of endothelial cells, fibroblasts, and surrounding ECM, respond like fibroblasts by upregulating TN on attached collagen gels. PCNA immunostaining was performed and showed a significant induction of proliferation in PAs from MCT-injected rats compared with saline-injected controls (Figure 7). The number of medial proliferating cells progressively increased on attached collagen (>2-fold by day 8) (P<0.05), whereas PAs cultured on floating collagen showed minimal evidence of proliferation, with PCNA values similar to those of saline controls (P<0.05).

We next sought to determine, by performing TUNEL assays, whether apoptosis was related to the regression in medial hypertrophy and suppression of TN in these hypertrophied rat PAs when they were floated on collagen gels (Figure 8A through 8E). To confirm that the in situ TUNEL assays were not aberrantly identifying necrotic cells as apoptotic, lactate dehydrogenase assays were performed. No significant difference was detected between attached and floating cultures and the tissue-free control (data not shown), indicating that the observed vascular regression was not due to the onset of necrosis. Normalized counts of medial apoptotic cells revealed low levels of apoptosis in MCT- and saline-injected rat PAs (Figure 8A, 8B, and 8E). When MCT-injected rat PAs were cultured on attached collagen, there was minimal or absent apoptosis (Figure 8C and 8E). Conversely, floating cultures displayed an early and sustained >6-fold induction of medial apoptosis from day 3 (P<0.05) (Figure 8D and 8E). In addition, normotensive rat PAs (saline injected) did not show an induction of apoptosis on either attached or floating gels (data not shown).

**Discussion**

In this study, we have shown that SMCs in the intact vessel, in the presence of endothelial cells, fibroblasts, and surrounding ECM, respond to mechanical changes in collagen gels. When the vessels are maintained on attached collagen gels, MMP and TN production and cellular proliferation increase, but, as a consequence of altered stress or deformation of the SMCs on floating gels, a reduction in MMP and TN expression and induction of apoptosis occur. The features described above are amplified when hypertrophied vessels are cultured in this fashion such that there is progression of medial wall thickening over time on attached collagen and regression on floating collagen gels. This model has extended observations related to cultured SMCs in collagen gels by revealing how coordinated loss of cellularity and ECM can structurally alter a blood vessel.

Organ cultures, used to study the behavior of aortic SMCs in their “native” environment, have never been applied to hypertensive arteries or to examine the evolution of structural changes in PAs. Here we show a model of PA organ culture that is faithful to clinical and experimental observations related to TN expression and SMC proliferation in the pathophysiology of remodeling and confirm our cell culture observations, indicating the underlying importance of MMP-2 activity. We further document that the “reverse process” results in regression of medial hypertrophy.

Previous studies have shown that TN is upregulated in hypertensive rat arteries and in response to angiotensin II. We have linked the changes in PA organ culture observed on attached and floating collagen gels to the effects of mechanical stress or deformation. These conditions affect cell shape, which is well established as an important determinant of cellular function. Vascular cells are naturally poised to respond to mechanotransduced signals from the ECM as a result of changes in hemodynamic forces. Studies in our laboratory have confirmed that vascular SMCs respond like fibroblasts by upregulating TN on attached collagen gels. Here we show that the increase in TN is associated with heightened mRNA levels. A putative “stress response sequence” in the TN promoter was identified in fibroblasts but does not appear to be the region in SMCs that is responsive in attached cultures.

In these organ culture studies, changes in TN expression were associated with MMP-2 and MMP-9 activity, being consistent with our previous studies showing that MMP-2 activity regulates TN synthesis in cultured SMCs. This mechanism, which involves MMP-mediated degradation of collagen, increases TN transcription via a mitogen-activated protein kinase pathway activated after SMC ligation with cryptic RGD sites exposed in denatured collagen. Conversely, inhibition of MMP-2 was associated with a reduction in TN expression and onset of apoptosis. Thus, there is a functional relationship between TN and MMPs, which codistribute at sites of vascular pathology.

Because MMP-2 regulates TN on floating collagen gels, it remains to be established how induction of MMP-2 might be related to alterations in mechanical stress or deformation. It is possible that the gene for this enzyme is mechanoresponsive or that MMP-2 is induced by serum or endothelial factors that penetrate into the subendothelium after mechanical perturbation of the endothelial surface, as previously proposed for induction of elastase activity in SMCs.

Our experiments using hypertrophied rat PAs documented a relative increase in elastase activity in cultures on attached...
versus floating collagen gels. As hypertensive vessels exhibit elevated serine elastase activity, these matrix proteinases might contribute to the pathogenesis of medial hypertrophy in organ culture by a TN-dependent pathway. Elastases may direct the upregulation of endogenous TN directly, through growth factor liberation, or indirectly, through MMP activation or increased expression.

The impact of MMP modulation of TN on SMC proliferation has been shown in these organ culture studies, and some of the mechanisms involved have previously been addressed in cell culture. TN acts as a critical SMC survival factor, which functionally amplifies the SMC proliferative response to liberated growth factors. Priming of growth factor receptors in this way occurs through their clustering at focal adhesion contacts formed by $\alpha_v\beta_3$-mediated interaction of SMCs with TN and cytoskeletal reorganization.

Our studies have also supported evidence showing that withdrawal of TN leads to apoptosis. The mechanism, which is largely unexplored, may be related to the initiation of "death gene"-inductive intracellular signals after the unmasking of integrins, particularly $\beta_3$, in the presence of growth factors. Studies have indicated that vascular cell survival and survival-related signals are mediated by ligation of the $\alpha_v\beta_3$ integrin receptor, which is the receptor for TN on SMCs. Indeed, $\alpha_v$ knockout mice die during embryonic life. This profound response, together with reports that TN

Figure 8. Apoptosis was monitored by TUNEL assay in PA organ culture tissue. A and B, Nuclei from the PAs of MCT-injected (A) and saline-injected control (B) rats after 21 days appear normal as stained by propidium iodide. There were few TUNEL-positive cells in PA tissues on attached collagen gels in organ culture as shown on day 3 (C) and as quantified in panel E. With regression of medial thickness, apoptotic cells were identified as bright green fluorescent nuclei beginning on day 3 (D) and are quantified in panel E. Bar=25 $\mu$m. Quantification of the percentage apoptotic cells at each time point indicates that these observed differences were statistically significant (E). Data are mean±SEM of 3 vessels at each time point. *$P<0.05$ comparing attached and floating conditions; †$P<0.05$ compared with day 0.
knockout mice exhibit only a very mild phenotype,\textsuperscript{41,42} suggests that this receptor may be critical for signaling of a number of alternative matricellular proteins. Our recent unpublished data (1998–1999) indicate that TN suppression within the vasculature is accompanied by upregulation of an alternative $\alpha_\beta_3$ ligating cell survival factor, osteopontin. Although embryonic vessel formation is inhibited with an $\alpha_\beta_3$ functional blocking antibody, it is interesting that this response is selective, in that established vessels do not require survival signals provided by $\alpha_\beta_3$-ECM interaction.\textsuperscript{35,43} This may explain the amplification of the MMP-TN-mediated effect in hypertrophied PAs, as SMCs in these actively remodeling vessels may be more dependent on $\beta_3$ integrin signals compared with normal PAs in which SMCs receive viability signals through other receptors.\textsuperscript{10,44,45}

Previous reports documenting a reduction in vascular wall thickness have focused solely on loss of cellularity,\textsuperscript{46,47} whereas a coordinated depletion of both cells and ECM is likely required for an optimal response. Progression of medial hypertrophy in PAs on attached gels was associated with an increased number of elastic laminae. It might be expected that an upregulation of ECM synthesis results from an increase in SMC cellularity and phenotypic modulation to a synthetic state. Indeed, our previous studies have shown high elastin turnover during progression of PA hypertrophy and have implicated increased elastase activity, consistent with our present findings.\textsuperscript{3,4} The mechanism appears to be related to elastin peptide induction of elastin synthesis, as demonstrated in fibroblast culture.\textsuperscript{48}

Conversely, SMC apoptosis is associated with loss of elastin. This is a particularly intriguing finding, because it is associated with suppression of classical ECM degrading enzymes, MMP-2, and elastases. In regression of PA medial hypertrophy after removal of rats from a hypoxic environment, increased expression of mast cell collagenase has been reported,\textsuperscript{49} however, a cause-and-effect relationship has not been determined. Because loss of elastin occurs under conditions of reduced proteinase activity in our model, we speculate, on the basis of ultrastructural studies,\textsuperscript{50} that elastin, and other ECM constituents, are being phagocytosed by vascular cells and degraded intracellularly. Alternatively, apoptosis-associated membrane permeability changes\textsuperscript{51,52} may result in activation of cell surface enzymes that proteolyze the ECM in the microenvironment.

In conclusion, we present a model whereby diseased PAs, which progressively hypertrophy on attached collagen, selectively undergo vascular regression on floating gels related to a reduction in proteolytic activity of both MMP-2 and elastases, downregulation of TN, suppression of proliferation, and induction of apoptosis. We suggest that progression versus regression of PVD is thus dependent on appropriate perturbation of fundamental cell-matrix interactions. Effective vascular lesion regression involves depletion of both ECM and cells, and a therapeutic strategy should ideally be directed at both of these components of the vessel wall.

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