Physiol. Res. 49: 577-585, 2000

Role of Proteolysis and Apoptosis in Regression of Pulmonary Vascular Remodeling

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Received February 22, 2000
Accepted April 3, 2000

Summary

Remodeled pulmonary arteries return to normal structural conditions after the increase in pulmonary artery flow resistance is reversed. We studied whether proteolysis of extracellular matrix proteins and apoptosis occur during reversal of remodeling produced by chronic hypoxia in the rat. Main pulmonary arteries were removed at different times during a 10-day period of exposure to 10% O_2 and 14 days after return to air. Content and rates of degradation of collagen and elastin as well as immunoreactive collagenase in tissue and isolated mast cells were measured. Immunoblots for collagenase and tissue inhibitor of metalloproteinases (TIMP) were performed. Apoptosis was assessed by cleavage of DNA and TUNEL assay. Excess collagen and elastin present at 10 days of hypoxia decreased to near normal levels after 3-5 days of air. Transient increases in collagenolytic and elastolytic enzyme activities accompanied the rapid decrease in matrix proteins. Mast cells containing collagenase accumulated in remodeled pulmonary arteries, and the active form of collagenase appeared at the time of peak proteolytic activity. TIMP increased during remodeling. Apoptosis was maximal 3 days after return to air. Our results suggest that activation of enzymes, which degrade matrix proteins, and apoptosis play a role in resolution of vascular remodeling.

Key words

Vascular remodeling • Apoptosis • Matrix metalloproteinase • Hypertension • Pulmonary

Introduction

A considerable amount of information is known about the cellular and molecular mechanisms that control pulmonary vascular remodeling (Stenmark and Mecham 1997). Little attention has be given, however, to the mechanisms which produce regression of pulmonary vascular remodeling after withdrawal of the stimuli which produce remodeling. Several lines of evidence clearly show that pulmonary arteries have the capacity to return to normal architecture. In animal models, the remodeling that occurs during chronic hypoxia is reversible over days to weeks after return to normoxia as shown by reduced thickness of pulmonary arteries (Herget *et al.* 1978, Fried and Reid 1984). In humans, there are a few reports of reversal of remodeling following surgical repair of mitral stenosis and congenital heart disease (Ramírez *et al.* 1968, Dammann *et al.* 1961). In the rat model of hypoxic

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pulmonary hypertension, we have observed ~2-fold increases in collagen and elastin content in the main pulmonary arteries following a 10-day exposure (Poiani *et al.* 1990). Remarkably, the content of these insoluble proteins decreased to control levels within 3-5 days of return to normoxia, suggesting that a mechanism involving proteolysis may contribute to regression. We have begun to explore the processes that lead to reversal of the remodeled pulmonary arteries. Accumulation of extracellular matrix proteins during remodeling occurs as a consequence of both an increase in the number of fibroblasts and smooth muscle cells and an increase in production of matrix proteins by these cells when they are in an activated state (Stenmark and Mecham 1997). Resolution of increased cell mass could be associated with reversal of the activated state or by a change in balance of cell death over cell proliferation resulting in a net loss of matrix-producing cells. A major process leading to loss of redundant cells is apoptosis, which is emerging as one of the mechanisms that regulates development and maintenance of normal vessel architecture (Dzau and Horiuchi 1998). We hypothesized that apoptosis in the pulmonary artery occurs during early regression of remodeling and also contributes to regression of remodeling.

Methods

The methods related to proteolysis have been described in detail (Tozzi *et al.* 1998, Thakker-Varia *et al.* 1998) and will be summarized here.

Animals

Groups of Sprague-Dawley rats (body weight 200-350 g) were studied prior to exposure (day zero), after hypoxia (10% O_2) for 10 days, or followed by recovery in air for 3, 7 or 14 days (referred to as days 13, 17 and 24). For some studies, rats were studied at 3 and 5 days of hypoxia and 1 day of recovery (day 11). Rats were anesthetized (pentobarital, 50 mg/kg intraperitoneally), and mean right ventricular pressure (RVP) was measured after the animals had breathed room air for 20 min (Kerr *et al.* 1987), a time sufficient to reverse hypoxic vasoconstriction in chronically hypoxic rats (Reid 1986). After sacrifice with an overdose of anesthetic, the ratio of the weights of cardiac ventricles (RV/[LV+S]), hematocrit, and the thickness of pulmonary arterioles (20-120 μm) were measured as previously described (Kerr *et al.* 1987). The main pulmonary artery was excised and prepared for biochemical, RNA or immuno-histochemical studies.

Biochemical and RNA assays

Biochemical analyses for hydroxyproline (an index of collagen) and desmosine (an index of elastin) were performed to assess the content of connective tissue proteins (Poiani *et al.* 1990). Collagenolytic activity was measured in homogenates of pulmonary arteries by incubating with guinea pig skin [^{14}C] type I collagen and elastolytic activity using [^{14}C] labeled bovine nuchal ligament elastin (Tozzi *et al.* 1998). Immunoblot analyses was performed on pulmonary artery homogenates with antibodies to rat collagenase and murine tissue inhibitor of metalloproteinase-1 (TIMP-1) (Tozzi *et al.* 1998). For RNA analysis, total RNA was extracted, and northern blot analysis was performed (Poiani *et al.* 1990). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with of a pair of oligonucleotide primers, 5′-GACCTCATGTTCATCTTTAG-3′ and 5′-CACCACAA TAAGGAATTCGT-3′, complementary to the rat collagenase cDNA (Quinn *et al.* 1990). The products were amplified and analyzed by Southern blot analysis.

Immunohistochemistry

Lung tissue was fixed by perfusion and sectioned as previously described (Tozzi *et al.* 1998). Tissue sections were incubated with nonimmune serum to block Fc receptors, reacted with antibody to rat interstitial collagenase (Quinn *et al.* 1990), and with a secondary antibody conjugated with rhodamine. As indicated in Results, the density of mast cells was increased in pulmonary arteries and to contain immunoreactive collagenase. We isolated peritoneal mast cells and stained them for collagenase since too few mast cells could be isolated from pulmonary arteries (Tozzi *et al.* 1998). Lung and peritoneal mast cells are connective tissue-type mast cells (Gibson and Miller 1986) and should express the same proteinases.

Apoptosis

Apoptosis in pulmonary artery tissues was evaluated by DNA fragmentation. Localization of apoptotic cells was performed by *in situ* staining of dying cells. Freshly isolated pulmonary artery tissue (~12-18 mg) was used for extraction of DNA. DNA fragmentation was evaluated by staining with ethidium bromide (Eastman 1995) (TACS Apototic Laddering kit, Trevigan, Gaithersburg, MD, USA). Characteristic 180

bp and multiples were used to identify DNA fragmentation. For staining of dying cells, *in situ* labeling of DNA breaks in individual nuclei in tissue sections was performed by the terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick-end labeling (TUNEL) method (Ben-Sasson *et al.* 1995) using a commercial kit (Apoptag Peroxidase, Trevigan). For quantitation, we counted labeled cells using a graticule in 6-10 areas of cross sectional cuts of main pulmonary arteries (~1000/vessel), and the results were expressed as percent of total cells.

Statistical analysis

Data analysis was by ANOVA (SAS 1982) with a $P<0.05$ considered significant.

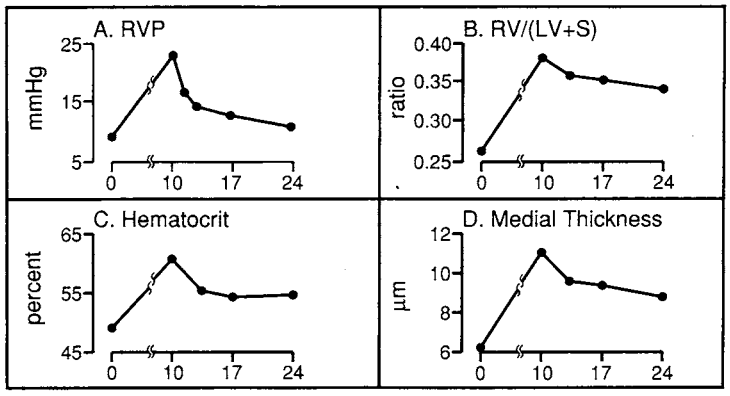

Fig. 1. *Time course of the regression of pulmonary hypertension. Groups of rats were exposed to air (day zero), 10% O_2 (hypoxia) at ambient pressure for 10 days, or hypoxia for 10 days and allowed to recover in air for 1, 3, 7 or 14 days (designated days 11, 13, 17 and 24, respectively).* ***A****. Mean right ventricular pressure (RVP).* ***B****. Ratios of weights of the right ventricle to left ventricle plus septum (RV/[LV+S]).* ***C****. Hematocrit.* ***D****. Medial wall thickness of muscular pulmonary arterioles. Data points represent mean values from 5-6 animals for each data point in panels A-C and from 4 animals for data in panel D. (Modified from Tozzi et al. 1998).*

Results

Pulmonary hypertension

Pulmonary hypertension and vascular remodeling were established by day 10 of hypoxic exposure as shown by increases in right ventricle pressure (RVP), RV/(LV+S) ratio and wall thickness of muscular pulmonary arteries (Fig. 1). These changes reversed within 3-5 days, as indicated by the reduction in RVP, RV/(LV+S), and medial wall thickness of muscular pulmonary arterioles compared to 10-day hypoxia (Fig. 1).

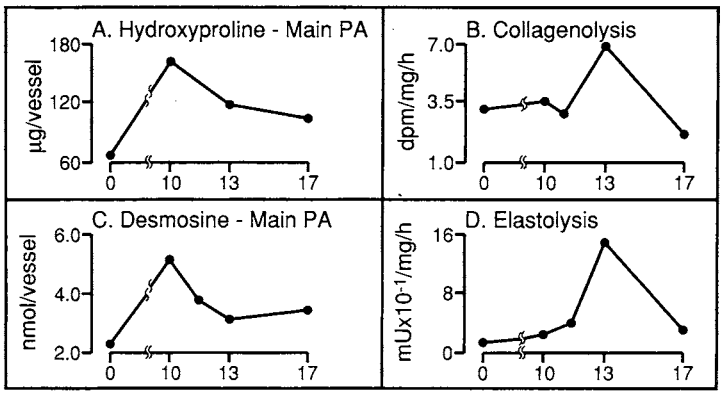

Fig. 2. *Biochemical changes in pulmonary arteries. Exposure conditions and designations of animal groups are described in Figure 1.* ***A****. Hydroxyproline content.* ***B****. Collagenolytic activity.* ***C****. Desmosine content.* ***D****. Elastase-like activity. Data points are mean values from 6 animals for each data point. (Modified from Tozzi et al. 1998).*

Biochemical analyses

Hydroxyproline content of main pulmonary artery was increased by ~2.5-fold at day 10 and was reduced at days 13 and 17 (Fig. 2A). Collagenolytic activity was increased 2-fold at day 13, a time corresponding to the rapid decrease in hydroxyproline

content (Fig. 2B). Desmosine content increased by ~2.5-fold at day 10 and was decreased on day 13 (Fig. 2C). A 7-fold increase in elastolytic activity was noted on day 13 (Fig. 2D), the time of decreasing desmosine content. Elastolytic activity was primarily of the serine protease type, as shown by >50 % inhibition by serine protease inhibitors (data not shown).

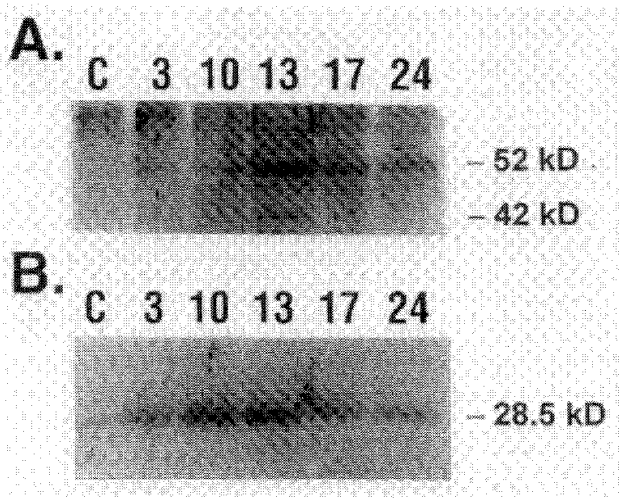

Fig. 3. *Autoradiograms of immunoblots for collagenase and TIMP-1. (A) Collagenase. (B) TIMP-1. Homogenates of pulmonary arteries (15 μg protein) were examined during hypoxia and recovery from hypoxia. Lanes indicate samples obtained from animals exposed to air (C), exposed to hypoxia for 3 or 10 days (3, 10), or exposed to hypoxia for 10 days and allowed to recover in air for 3, 7 or 14 days (13, 17, 24). Results of six pooled samples are shown at each time. In panel A, the higher M_r form of collagenase (~52 kDa) representing latent collagenase was present at all times during hypoxia. The lower M_r form of collagenase (~42 kDa) representing active enzyme was present on day 13. In panel B, TIMP-1 protein is faintly visible in C, was increased in days 10 and 13, and returned toward controls on days 17 and 24. (Reproduced with permission from Tozzi et al. 1998).*

RNA levels

Messenger RNA levels for collagenase and TIMP-1 were not detected by northern blot analysis under low stringency conditions nor were RT-PCR products detected. These results indicate the presence of collagenase and TIMP-1 gene products were below detectable levels during pulmonary artery remodeling and suggest that the mechanism of increased collagenase and TIMP-1 were regulated by post-transcriptional mechanisms.

Immunoblots

The inactive form of collagenase (~52 kDa) was detected by immunoblot during hypoxia, and the active form of collagenase (~42 kDa) was detected only at day 13, the period of peak collagenolytic activity (Fig. 3A). This finding suggests that activation of collagenase from a latent to an active form may contribute to collagen degradation in the pulmonary artery during recovering from hypoxia. TIMP-1 (~28.5 kDa) increased during hypoxia and reached the highest level at days 10-13 (Fig. 3B).

Immunohistology

We observed a striking difference in the localization of immunoreactive collagenase in control and hypertensive pulmonary arteries. In controls, there was a diffuse pattern of staining in muscular pulmonary arterioles (Fig. 34A). At 10 days of hypoxia and after one day of recovery (day 11), fluorescence was associated with granular cells in the vessel wall (Figs 4B and 4C). At days 13, 14, and 24, the earlier granular fluorescence was decreased (Figs 4D, 4E and 4F). The granular pattern in hypertensive vessels was explained by the predominate localization of collagenase in connective tissue-type mast cells, as shown by colocalization of collagenase and mast cell chymase I (Tozzi *et al.* 1998). The density of mast cells in pulmonary arteries increased during hypoxia, as shown by the increasing percentage of mast cells on the following days: zero (1.0±0.2 %), 10 (2.6±0.7 %), 13 (4.1±0.9 %) and 17 (2.0±0.3 %) (n = 5-6 animals per group; all $P<0.001$ vs. day zero). Since too few lung mast cells were isolated for analysis, peritoneal mast cells (≥96% purity) were used to test for the presence of collagenase and mast cell chymase I proteins. Both proteins were identified in peritoneal mast cells (Tozzi *et al.* 1998). Thus, connective tissue-type mast cells contain collagenase, and mast cell density increases in pulmonary arteries during hypoxia. It appears that the major source of collagenase protein in pulmonary arteries during remodeling is mast cells.

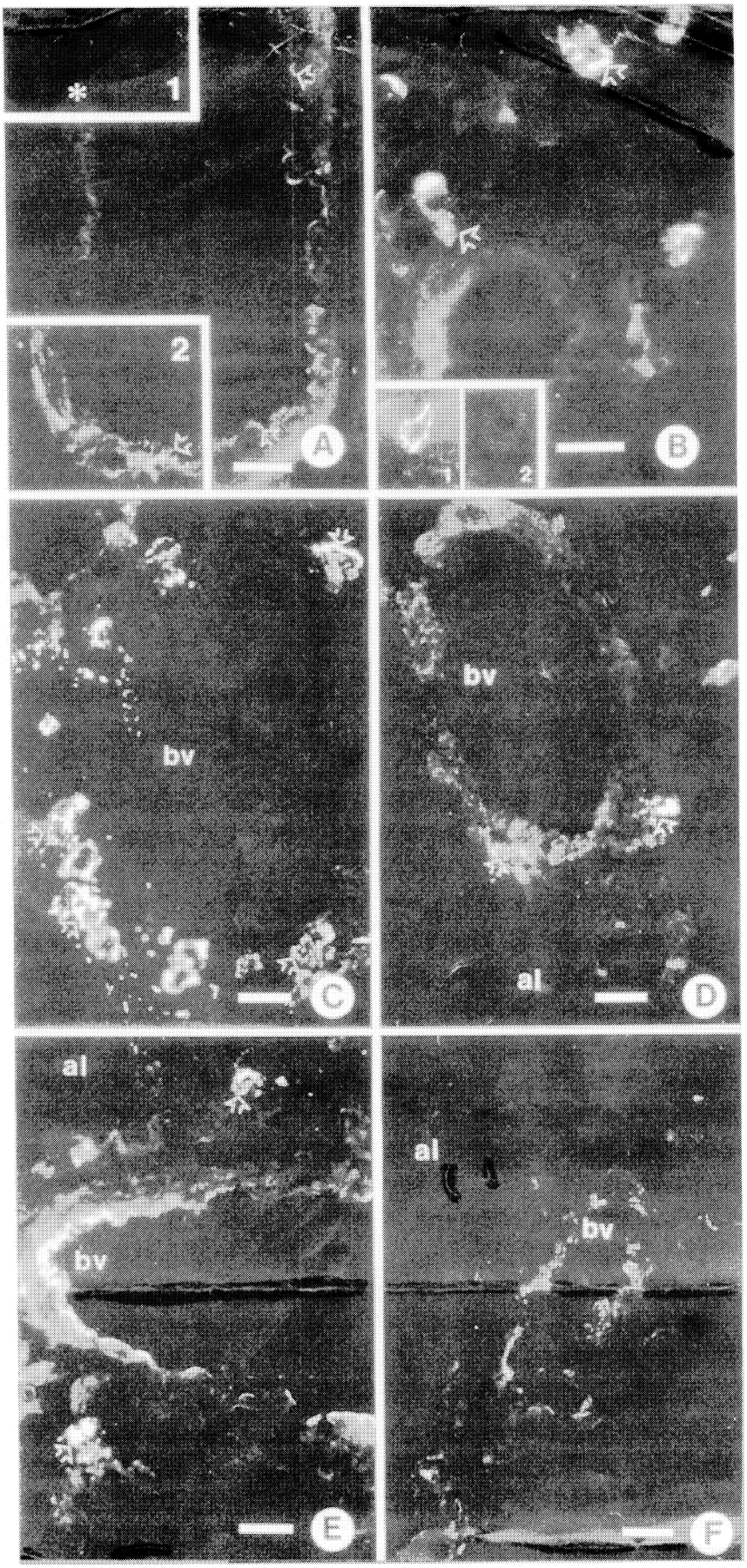

Fig. 4. *Localization of collagenase in pulmonary arteries.* ***A.*** *Control pulmonary artery. Arrows indicate rhodamine immunofluorescence of collagenase located subendothelially adluminal to the internal elastic lamina in a muscular pulmonary artery (180 μm external diameter). Note diffuse fluorescent staining the media. Bars in all panels represent 20 μm. Inset 1. Portion of wall of muscular pulmonary artery (asterisk indicates the lumen) treated with PBS and rhodamine-labelled secondary antibody. Weak non-specific fluorescence is detected. x450. Inset 2. Texas red immunofluorescence localization of desmin in same tissue showing antibody staining in the smooth muscle cells of the media (arrows). x450.* ***B.*** *Ten-day hypoxia. Arrows indicate fluorescence associated with granules and granular cells in the vessel wall.* ***C.*** *Day 11. Open arrows indicate locations of procollagenase antibody deposition in granules in the media and adventitia of the blood vessel (bv) wall.* ***D.*** *Day 13. Open arrows indicate sites of antibody deposition. An alveolus (al) is shown for reference.* ***E.*** *Day 17.* ***F.*** *Day 24. Procollagenase localized diffusely in media with some deposition in subendothelial region. Earlier granular fluorescence was decreased at days 13, 17, and 24.*

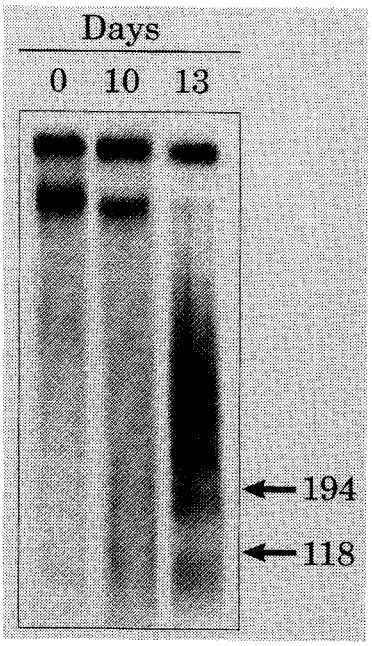

Fig. 5. *DNA digestion in homogenates of pulmonary artery. Pulmonary arteries from an air-exposed group (day 0), a group exposed to hypoxia for 10 days (day 10), and exposed for hypoxia for 10 days and to air for 3 days (day 13) were used. DNA digestion was analyzed by electrophoresis. Arrows and numbers indicate standard molecular weight size markers.*

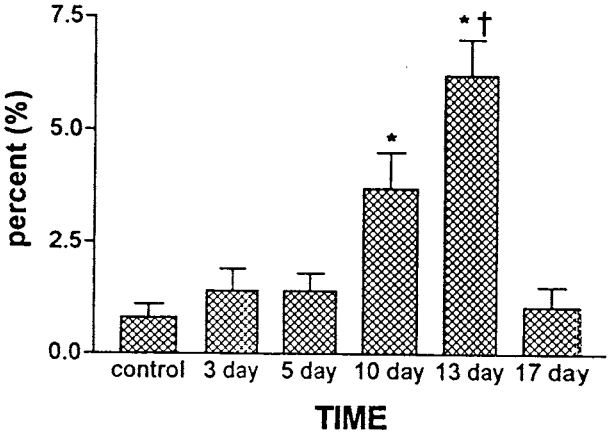

Fig. 6. *Percentage of apoptotic cells in pulmonary arteries. Main pulmonary arteries were prepared from air-exposed animals (control), animals exposed to hypoxia for 3, 5, or 10 days, or animals exposed to hypoxia for 10 days and returned to air for 3 or 7 days (days 13 and 17). Apoptosis in tissue specimens was identified by specific staining of DNA fragmentation with terminal deoxynucleotidyl transferase (TdT), TdT/dUTP-biotin nick-end labeling (TUNEL) staining. Results were expressed as percentage of total cells. Data are mean ± S.E.M. (n=6-9; *, P<0.05 vs. control; †, P<0.05 vs. 10 day).*

Apoptosis

Apoptosis in pulmonary arteries during the early period of regression was investigated as a mechanism to explain the decrease in cellularity that occurs during regression of pulmonary artery remodeling. We measured fragmentation of total DNA extracted from main pulmonary arteries at days 0, 10 and 13 (Fig. 5). Characteristic fragmentation of DNA into ~180 bp fragments were noted at day 13, but little or no fragmentation was observed at days 0 and 10. The percentage of apoptotic cells was quantitiated by counting apoptotic cells identified by TUNEL assay and expressing the results were expressed as percentage of total cells (Fig. 6). We found a significant increase in percent apoptotic cells at day 13 compared to day 10, and there was a significant increase in apoptosis at day 10 compared to controls. These data suggest that apoptosis occurred at day 13 and may have contributed to the decrease in cells in the remodeled pulmonary arteries.

Discussion

Our results show that relatively marked remodeling of central pulmonary arteries can undergo restoration to normal over a relatively short period of time. We observed marked increases in activities of collagenolytic and elastolytic enzymes that occurred at the same time as contents of collagen and elastin were decreasing during the early recovery period. The elastotolytic enzyme is predominately a serine protease but has not been characterized. Serine proteases have been identified in pulmonary arteries in monocrotaline-induced pulmonary hypertension (Zhu *et al.* 1994). It is possible that the elastolytic enzyme present during recovery of hypoxic pulmonary hypertension is one of these serine proteases. The major extracellular pathway of collagen resorption is through the matrix metalloproteinases (MMPs), a family of Zn^{2+}- and Ca^{2+}-containing enzymes (Matrisian 1990). Collagenases, stromelysins and gelatinases act together and can completely degrade matrix components. These enzymes are activated from latent to active forms by cleavage of proenzyme forms of the molecules to active

forms. On day 13 we identified the presence of a ~52 kDa band of inactive collagenase on immunoblots in hypertensive pulmonary arteries and the presence of a ~42 kDa band consistent with the activated form of collagenase (Roswit *et al.* 1983). We have found the activation of MMPs other than collagenase during early regression of hypoxic remodeling as shown by increased protein levels or activities of stromelysin-1 and the ~72-, ~68-, and ~60-kDa gelatinases (Tozzi *et al.* 1998, Thakker-Varia *et al.* 1998). The extracellular matrix protein turnover is controlled by TIMPs and, less specifically, by α_2-macroglobulin that together with MMPs form a tightly regulated system. TIMP-1 is an inducible protein that increases during remodeling. Although TIMP-1 levels increased, it is difficult to determine the specific role of TIMP-1/MMP balance in degradation of extracellular matrix components because of sequestration of enzymes, kinetics of enzyme-inhibitor binding, and the turnover rates of both proteins. Although these experiments show a temporal correlation between MMP activities and decreases of matrix protein, they do not establish causality. Additional experiments such as *in vivo* administration of protease inhibitors are needed to demonstrate that proteolytic enzymes modulate vascular remodeling.

Increased mast cell density has been noted in a variety of conditions associated with pulmonary hypertension, including chronic hypoxia (Tucker *et al.* 1977) and pressure-induced right ventricular hypertrophy (Oliveti *et al.* 1989) in rats and plexogenic pulmonary arteriopathy in humans (Heath and Yacoub 1991). The pathological significance of these observations are obscure. We observed an association of immunoreactive collagenase with connective tissue-type mast cells in the remodeled pulmonary arteries and showed that peritoneal mast cells, which are phenotypically identical to those in the pulmonary artery, also stain with collagenase. The mechanism by which mast cells increase in pulmonary arteries is not known. The absence of collagenase gene products suggests that *de novo* synthesis of collagenase by resident mast cells did not occur. Possible explanations are either recruitment of mast cells containing preformed collagenase from precursors in bone marrow (Galli 1993) or uptake of collagenase from local vascular cells since dermal mast cells have been shown to take up collagenase from other cells (Krejci *et al.* 1992). Thus, our data suggest that mast cells may be key effector cells in mediating collagen turnover, but clearly more studies are needed to establish their role in vascular remodeling.

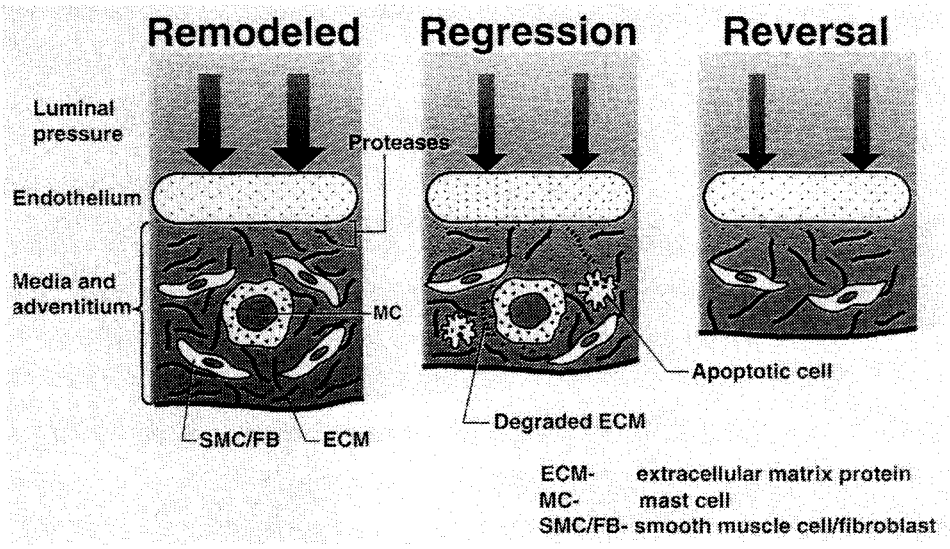

Fig. 7. *Scheme of events during regression of pulmonary artery remodeling. In remodeled pulmonary arteries, the media and adventitium of blood vessels is thickened by increased smooth muscle cells (SMCs) and fibroblasts (FBs) as well as extracellular matrix proteins (ECM). Mast cells (MC) are increased in density. Proteases are secreted from endothelial cells at a basal rate. During regression, pulmonary artery pressure is lowered, the level of proteases from endothelium is increased, and extracellular matrix proteins undergo degradation. In addition, vascular cells undergo apoptosis. During reversal of pulmonary hypertension, the thickness of the vessel wall has returned toward normal, there are fewer cells in the media and adventitium, and the level of proteases has returned to baseline.*

We explored whether vascular cells were lost during an episode of pulmonary artery remodeling by apoptosis and whether the peak period of apoptosis correlated with proteolysis. Apoptosis was demonstrated in main pulmonary artery homogenates by endosomal cleavage of DNA, an essential component of apoptosis (Eastman 1995). Since nucleosomal DNA cleavage is qualitative rather than quantitative, the TUNEL method was used which relies on the *in situ* labeling of DNA breaks in individual nuclei, and labeled nuclei in tissue section were counted in separate groups over time. Our results show that ~1-2% of cells in control vessels are TUNEL-positive and that the percentage of apoptotic cells remains unchanged during the period of rapid cell growth and hypertropy on days 3 and 5. At day 10 when the vessel is remodeled increased apoptosis was present, presumably to bring into balance growth and death of cells as part of the structural equilibrium in established pulmonary hypertension. During recovery (day 13), a further rise in percentage of apoptotic cells was noted, apparently reflecting a shift in the balance towards cell death as remodeling resolves. Similar temporal patterns of removal of cells and matrix components were present, consistent with "physiologic" involution of vessels.

As outlined in Figure 7, we propose that changing conditions in the blood vessel wall trigger an apparent shift in the steady-state toward resolution of remodeling. Changing hemodynamic conditions which are relayed *via* endothelium are proposed as the primary stimulus. This concept is based on preliminary *in vitro* studies showing that reducing the tension on isolated segments of remodeled pulmonary arteries elicits proteolysis and apoptosis and the process is dependent on an intact endothelium (Tozzi *et al.* 1993, 1999). Unknown endothelial-derived substances may act in a variety of ways to control the homeostasis of connective tissue and cells in the vessel wall. One process may involve signals from endothelium which stimulate mast cells to release MMPs and lead to matrix degradation. In adaptive remodeling such as pulmonary artery remodeling, coordinate processes may control matrix proteolysis and apoptosis. For example, a common mediator and/or signaling pathway may exist for proteolysis and the genetic program for apoptosis. Alternatively, a cell population may exist within blood vessels that both expands and undergoes cell death in response to changing signals during remodeling. Another possibility is that apoptosis of selected cells is regulated by proteolysis which acts by disrupting cell-matrix attachments, thus providing a common mechanism for reducing matrix and cells. Future studies of resolution of vascular remodeling will doubtless involve a dynamic interplay between hemodynamic forces, locally generated signaling molecules, matrix degradation, and cell-specific apoptosis.

Acknowledgements

The authors thank Guanghua Chen, Rena Bannett, Bonnie Peng, Linda Tatum, and Joanne Babiarz for technical assistance and Mrs. Charmayne Fleming for secretarial services. Dr. S-Y. Yu of the University of Rochester School of Medicine and Dentistry performed the desmosine assays. This work was supported by NIH grants HL24264, the Barbara Cornwall Wallace Respiratory Research Laboratory, and the Medical Research Service of the Department of Veterans Affairs.

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