Expression of Extracellular Matrix Proteins Accompanies Lesion Growth in a Model of Intimal Reinjury

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Abstract—Reinjury of rat arterial lesions induces an increase in lesion size that is not associated with an increase in cell number. In this study, matrix volume was examined after reinjury to preexisting lesions, and the kinetics of matrix gene expression and activity of proteolytic enzymes in the lesion were evaluated. Volume densitometry in intima showed a significant increase in matrix volume 28 days after the reinjury, although no change was observed at 14 days. Three common vascular matrix molecules, \( \alpha_1(1) \)procollagen, tropoelastin, and fibronectin, were expressed highly at 7 days after the reinjury. Expression of tropoelastin remained upregulated for the entire 28 days after the reinjury, whereas \( \alpha_1(1) \)procollagen and fibronectin returned to the control level by 28 days. Protease activity was also increased after reinjury. Within days, a marked increase in urokinase plasminogen activator activity was observed in intima, and this activity decreased to control level by 14 days. The activity of tissue plasminogen activator did not change. The 95-kDa gelatinolytic activity was increased 1 to 2 days after the reinjury, but no change in other gelatinolytic activities was observed. These findings demonstrate that the accumulation of extracellular matrix is important in the increase in lesion size after reinjury and that a balance of matrix synthesis and degradation may explain why no change in matrix volume was detected until 28 days after the reinjury. (Circ Res. 1998;82:988-995.)

Key Words: angioplasty ■ extracellular matrix ■ plasminogen activator ■ matrix metalloproteinase

Intimal hyperplasia is thought to play a critical role in the development of restenosis in the atherosclerotic artery, but mechanisms promoting these lesions have been poorly understood. Animal models of arterial injury have significantly increased our understanding of how lesions develop, although thus far the data obtained from these experiments have not led to improved treatment of restenosis in humans. One possible reason is that most in vivo studies have examined the response of a normal healthy artery to mechanical injury, and restenosis is known to occur in previously diseased arteries. We previously studied the response of rat carotid arteries with preexisting lesions to angioplasty injury (reinjury model). We found that SMC replication was detected until 28 days after the reinjury. Within days, a marked increase in urokinase plasminogen activator activity was observed in intima, and this activity decreased to control level by 14 days. The activity of tissue plasminogen activator did not change. The 95-kDa gelatinolytic activity was increased 1 to 2 days after the reinjury, but no change in other gelatinolytic activities was observed. These findings demonstrate that the accumulation of extracellular matrix is important in the increase in lesion size after reinjury and that a balance of matrix synthesis and degradation may explain why no change in matrix volume was detected until 28 days after the reinjury.

Materials and Methods

Arterial Reinjury Model

Male Sprague-Dawley rats (B&K Universal, Kent, Wash), aged 3 to 4 months, were used in all experiments, and all surgery was performed under general anesthesia with an intraperitoneal injection of xylazine (Xyla-ject, 4.6 mg/kg body wt, Phoenix Pharmaceutical Inc) and ketamine (Ketaject, 70 mg/kg body wt, Phoenix Pharmaceutical Inc). Animals were subjected to reinjury of the left common carotid, as described previously. The left common carotid was injured three times with a 2F Fogarty catheter (Baxter Healthcare Co) introduced through the left femoral artery (first injury). At 28 days after the first injury, a reinjury or sham operation was performed. A 1.5-mm-diameter coronary dilation catheter (ACS TEN; balloon length, 20 mm; Advanced Cardiovascular Systems Inc) was introduced through the left external carotid, and the proximal edge of the catheter was positioned at carotid bifurcation. The injury consisted of two 1-minute inflations of the balloon at 4 atm with a 30-second interval (reinjury). For the sham operation, the left external carotid artery was exposed and ligated without dilation of the common carotid.

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For morphological analysis, rats were killed at 14 and 28 days after the reinjury and the sham operation. After death by overdose injection of sodium pentobarbital (intravenous Nembutal, Abbott Laboratories), animals were prepared by perfusion/fxation using 4% paraformaldehyde as described previously. The left common carotid was excised, and two segments of carotid between 5 and 10 mm from carotid bifurcation were used for transmission electron microscopy and volume density analysis, and segments between 3 and 5 mm from the bifurcation were used for quantifying intimal size. For RNA and protein extraction, rats were killed at various times after the reinjury and at 28 days after the first injury (control). One group of preoperative rats was killed to provide normal control media. Ten minutes before death by pentobarbital overdose, rats received an intravenous injection of Evans blue (200 μL of 5% solution, Sigma) to mark the deendothelialized area. Carotid arteries were briefly flushed with iced-cold lactated Ringer’s solution (Baxter Healthcare Co) at physiological pressure to remove blood, and the whole length of left carotid was excised. The reendothelialized carotids were opened longitudinally after removing the surrounding connective tissue. The neointima was then stripped (at the internal elastic lamina) from the adventitia. All specimens were then snap-frozen in liquid nitrogen and stored at −80°C.

**Transmission Electron Microscopy and Volume Density of Matrix in Intima**

The volume density of intimal ECM was determined by the method using a 108-point square lattice test grid on a transmission electron microscope.15 The excised arteries were immersed in phosphate-buffered 2.5% glutaraldehyde/2% paraformaldehyde for >24 hours. Three small pieces of artery were cut per animal (each 1 mm apart), postfixed in 1% OsO4 for 1 hour, stained en bloc in 2% uranyl acetate, and embedded in paraffin. The three 4-μm sections (each 1 mm apart) from the adventitia. All specimens were then snap-frozen in liquid nitrogen and stored at −80°C.

**RNA Extraction and Northern Blot Analysis**

Tissue samples from >7 animals were mixed together and prepared for each time point (total, 45 animals). Frozen arterial tissue was ground to a fine powder under liquid nitrogen, and total cellular RNA was isolated with Trizol (GIBCO BRL) according to the manufacturer’s instruction. The RNA concentration was measured by absorption at 260 nm. Equal amounts (5 μg of total RNA) of each extract were separated on formaldehyde agarose gel (1.2%) and transferred to nylon membranes (Zeta-Probe, Bio-Rad Laboratories), as described previously.16 After transfer, RNA blots were exposed to shortwave UV light and baked at 80°C for 2 hours to cross-link RNA to the membrane. The blots were prehybridized with hybridization buffer (50% formamide, 0.75 mol/L NaCl, 50 mmol/L Tris [pH 7.4], 1× Denhardt’s solution, 1% SDS, 10% dextran sulfate, and 200 μg/mL salmon sperm DNA) at 42°C for 6 hours and hybridized with hybridization buffer containing 32P-labeled cDNA probes (1.5×106 cpm/mL) at 42°C for 16 to 24 hours. The cDNA probes were labeled with [32P]dCTP by random primer extension (Multi-Prime, Amersham). After hybridization, the blots were washed in two changes of 2× SSC/0.1% SDS for 15 minutes at room temperature and in two changes of 0.3× SSC/0.1% SDS for 15 minutes at 65°C and then autoradiographed. To verify loading in each lane, the blots were probed with a labeled oligonucleotide directed to the 28S rRNA, with the sequence 5′ GGCGAGGGCCGACCTCATCGGAGC 3′.

**Zymography**

Arteries from 5 animals per each time (total, 35 animals) were pooled and pulverized under liquid nitrogen and incubated in ice-cold lysis solution (1% SDS, 50 mmol/L Tris [pH 7.6], and 1 mg/mL leupeptin) for 30 minutes while being pulled through a 23-gauge needle. Insoluble matter was removed by centrifugation, and the protein concentration was measured by bicinchoninic acid assay (Pierce). Lysates were incubated with sample buffer (1% SDS, 10% glycerol, 50 mmol/L Tris [pH 6.8], and 0.01% bromophenol blue [final concentration]) for 15 minutes at 4°C for zymographic assay of PAs and gelatinolytic enzymes or incubated with sample buffer containing 2.5% 2-mercaptoethanol for reverse zymography of PAI. To detect the activities of PAs and PAI, equal amounts (10 μg of total protein) of each sample were separated on 8% SDS-PAGE. The gel was soaked in two changes of 2.5% Triton X-100 (Sigma) for 45 minutes each time and in two changes of 100 mmol/L Tris (pH 8.1) for 30 minutes each time at room temperature before being layered on the substrate gels. For PA zymography, substrate gels consisted of a mixture of 1.25% agar, 2% nonfat milk, and 40 μg/mL plasminogen from human plasma (Sigma) in 100 mmol/L Tris (pH 8.1) on a flat glass, and 50 μg/mL urokinase (human high molecular weight, Calbiochem) was added to the substrate gel for reverse zymography to detect PAI activity.27 These zymograms were allowed to develop at 37°C for 3 to 24 hours, and photographs were
taken using dark-ground illumination. To estimate the gelatinolytic activity, equal amounts (10 μg of total protein) of each sample were applied onto 8% SDS-PAGE copolymerized with 0.1% gelatin (Sigma) as substrate. After electrophoresis, the gels were soaked in 2.5% Triton X-100 for 30 minutes at room temperature with one change of the solution, followed by overnight incubation at 37°C in incubation buffer (50 mmol/L Tris [pH 8.1], 2.5 mmol/L CaCl₂, and 0.02% NaN₃). To stop the reaction, the gels were washed in 10% trichloracetic acid (Baker) and then stained with rapid Coomassie stain (Diversified Biotech) for 30 minutes, destained in 30% methanol/10% acetic acid, and photographed.

**Western Blot Analysis**

Equal amounts (10 μg of total protein) of each lysate were incubated for 15 minutes at 4°C with sample buffer containing 2.5% 2-mercaptoethanol to detect PAI-1. Samples were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane (Protran, Schleicher & Schuell). Blocking, incubation with antibodies, washing, and detection by enhanced chemiluminescence (Amersham) were carried out according to the protocol described previously. The primary antibody used for the analysis was a rabbit polyclonal antibody against rat PAI-1 (American Diagnostica Inc).

**Statistics**

The difference in matrix volume density between reinjured rats and sham-operated rats was analyzed by unpaired Student t test, and the Pearson correlation coefficient was used to examine the correlation between intimal size and matrix volume density after reinjury. All data were considered significant at \( P<0.05 \).

**Results**

**Transmission Electron Micrograph and Volume Density of Matrix in Intima**

Our previous study on reinjury of rat arteries showed that there was an increase in intimal lesion size after 28 days that was not related to an increase in cell number. In the present study, transmission electron micrography revealed that intimal cell density was reduced in arteries at 28 days after the reinjury compared with sham-operated arteries (Figure 1). Furthermore, there was an abundance of elastic fibrils in the intercellular space 28 days after the reinjury (Figure 1). The volume density of intimal ECM was significantly increased 28 days after the reinjury (\( P=0.0003 \)), although no apparent change was detected at 14 days after the reinjury (\( P=0.054 \)) (Figure 2A). Furthermore, intimal size after reinjury was correlated significantly with the volume density of intimal matrix (\( r=0.792, P=0.0023 \)) (Figure 2B).

It should be noted that the volume density of the matrix was obtained from individual animals and average values are shown (Figure 2A). This is in contrast to the data presented below, for which it was necessary to pool tissues from different animals to determine gene expression or proteolytic activities.

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**Figure 1.** Transmission electron microscopy of carotid intima. Panel A is from a rat at 28 days after reinjury, and panel B is from a rat at 28 days after sham operation. A reduction in cell density can be seen in panel A. Bar=5 μm. E indicates elastin.

**Figure 2.** Panel A shows volume density of matrix in the intima after reinjury (solid bar) and sham operation (open bar). Note the significant difference between reinjury and sham operation at day 28, but not at day 14 (d indicates days after reinjury). Values are shown as mean±SD. Panel B shows correlation between intimal size and volume density in intimal matrix after reinjury. These two values are significantly correlated by linear regression. ○ indicates 14 days after reinjury; □, 28 days after reinjury.
Expression of Matrix and TGF-β1 Genes

The expressions of αI(1)procollagen, tropoelastin, and fibronectin genes were examined using Northern blot analysis. At 7 days after the reinjury, expression of mRNA for all three was increased in the intima (Figure 3). Expression of αI(1)procollagen returned to the control level by 14 days after the reinjury (Figure 3A and 3a), whereas the expression of tropoelastin was still elevated 28 days after the reinjury (Figure 3B and 3b). An increase in fibronectin expression was observed at 7 to 14 days after the reinjury, although the level was decreased by day 28 (Figure 3C and 3c). TGF-β1 expression in the intima was also upregulated at all times studied after reinjury (Figure 3D and 3d).

In the media after the reinjury, no significant change was observed in the expression of αI(1)procollagen, fibronectin, tropoelastin, or TGF-β1 (data not shown).

PA Activity and Expression

The above data show that an increase in matrix synthesis occurred at times after reinjury when no apparent change in matrix volume density was detected. One possible explanation for this findings is that the newly synthesized matrix was degraded. Therefore, we measured the activity of PAs and gelatinolytic enzymes in these arteries.

PA activity, assessed by zymography, showed an increase in UPA activity 1 day after the reinjury, but thereafter, the activity decreased gradually and returned to baseline by day 14 (Figure 4A). TPA activity in the intima, however, did not change (Figure 4B). In the media, UPA activity increased after reinjury in a pattern similar to that for the intima, and no significant change in TPA activity was detected after reinjury (Figure 4C).

Expression of UPA mRNA increased at 2 and 7 days after the reinjury in the intima and returned to the control level by day 14 (Figure 5A and 5a). In contrast, TPA expression in intima was downregulated at 2 days after the reinjury but returned to control level by day 14 (Figure 5B and 5b). In media, neither UPA nor TPA mRNA changed significantly after reinjury (data not shown).
fact, the SMC density of these lesions was significantly increased after reinjury. However, our results clearly showed that there is a significant increase in ECM volume 28 days after the reinjury. That this increase in matrix volume was detected at 28 days and not at 14 days correlates well with our finding that a significant increase in intimal size was detected 28 days after the reinjury, and a significant correlation between matrix volume and intimal size after reinjury was detected in the present study. These data also show that lesion growth in an artery with a preexisting intimal lesion is a late event compared with that observed in normal arteries, where an increase in intimal mass can be detected as early as 4 days after balloon catheter injury. Several studies of human restenosis specimens after PTCA have suggested that ECM played an important role in the development of restenotic lesions. Schwartz et al presented data showing that the cellular component of the intima was ECM. The increase in matrix volume density in these rat tissues would support these data. Serial coronary angiography after PTCA has revealed that restenosis developed between the first and third month after angioplasty, and it is interesting to note that the increase in these rat lesions was first observed at day 28. In the development of human lesions, there are several critical aspects not seen in the rat reinjury model, such as inflammatory cell infiltration, lipid deposition, and mural thrombus, and care must be taken in extrapolating our data with the progression of lesion growth in humans.

Since an increase in lesion size was detected 28 days after the reinjury, we would have predicted matrix molecules to be strongly expressed at late times (14 to 28 days) after the reinjury. Surprisingly, this was not found to be true, and our data show that although three common matrix molecules investigated in the present study were strongly expressed in the intima at 7 days after the reinjury, only tropoelastin was expressed above background levels after 28 days. Even allowing for a delay between matrix expression and our ability to detect any matrix accumulation, this result was unexpected. Therefore, we examined changes in expression and activity of proteolytic enzymes that are known to be expressed by rat arterial cells, since any accumulation of matrix is a balance between synthesis and its degradation. An increase in UPA mRNA was noted in the intima at 2 and 7 days after the reinjury, but the expression was downregulated to background levels by 14 days. Surprisingly, UPA activity was highest at day 1 and decreased thereafter. A similar discrepancy between UPA expression and activity has been observed in a normal rat carotid subjected to balloon injury, with no obvious explanation. One possibility is that the activity values of UPA are influenced by the presence of PAI-1. The activity of this PAI in the intima peaked at 4 days after the reinjury, which is the same time when UPA activity was decreased. One possibility, therefore, is that diminished UPA activity, despite an increase in mRNA, can be attributed to the presence of PAI-1.

TPA mRNA expression in the intima was downregulated at 2 and 7 days after the reinjury, but there was no change in its activity. The lack of concordance between activity and expression may be due to plasma-derived TPA. This could be because of net TPA synthesis by endothelial cells and an

PAI Activity and Protein Expression in Intima
Both UPA and TPA are blocked by PAI; therefore, we wished to determine whether this PAI was also regulated by reinjury. Reverse zymogram showed a lysis-resistant zone at 50 kDa, suggesting PAI activity (Figure 6A). This activity was detected at 2 days and reached its maximum at 4 days after the reinjury. No PAI activity was detected at day 14. A Western blot for rat PAI-1 also showed a 49-kDa protein whose expression was increased at day 2, with strong expression at day 4 (Figure 6B). PAI-1 was still detected by day 7 but not by day 14.

Gelatinolytic Activity
In the control vessel, four prominent bands of gelatinolytic activity were present in both the intima and media, with molecular masses of 55, 62, 70, and 88 kDa (Figure 7). At 1 and 2 days after the reinjury, an extra band of 95 kDa appeared in both the intima and media, but it disappeared by day 4 (Figure 7). No significant changes in other gelatinolytic activities were observed in either the intima or media (Figure 7). We believe that 88- and 95-kDa gelatinolytic activities represented active and latent forms of MMP-9, respectively. The 70-kDa activity corresponded to a latent form of MMP-2, and the 55- and 62-kDa activities probably represented its active form.

Discussion
In a previous study, we showed that injury of a preexisting intimal lesion stimulated an increase in lesion size, but this was not accompanied by an increase in SMC number. In fact, the SMC density of these lesions was significantly reduced. These data led us to propose that unlike the lesion growth that occurs after injury to normal rat arteries, the growth of lesions in arteries with preexisting intimal lesions is due to cell matrix accumulation and not to an increase in cell number. One aim of the present study was to provide evidence to support this hypothesis, and our results clearly show that there is a significant increase in ECM volume 28 days after the reinjury. That this increase in matrix volume was detected at 28 days and not at 14 days correlates well with our finding that a significant increase in intimal size was detected 28 days after the reinjury, and a significant correlation between matrix volume and intimal size after reinjury was detected in the present study. These data also show that lesion growth in an artery with a preexisting intimal lesion is a late event compared with that observed in normal arteries, where an increase in intimal mass can be detected as early as 4 days after balloon catheter injury. Several studies of human restenosis specimens after PTCA have suggested that ECM played an important role in the development of restenotic lesions. Schwartz et al presented data showing that the cellular component of the intima was ECM. The increase in matrix volume density in these rat tissues would support these data. Serial coronary angiography after PTCA has revealed that restenosis developed between the first and third month after angioplasty, and it is interesting to note that the increase in these rat lesions was first observed at day 28. In the development of human lesions, there are several critical aspects not seen in the rat reinjury model, such as inflammatory cell infiltration, lipid deposition, and mural thrombus, and care must be taken in extrapolating our data with the progression of lesion growth in humans.

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increase in plasma concentrations. The fact that these lesions do not possess an intact endothelium would render the arterial wall permeable to plasma proteins, which would include TPA.\textsuperscript{31,36}

The presence of increased PA activity at early times (1 to 7 days) after reinjury may explain why no increase in matrix accumulation was detected at the early times after reinjury but does not provide an answer as to why an increase in ECM was detected 28 days after reinjury. In understanding this result, it is important to note that of the three common matrix molecules expressed after reinjury, only tropoelastin is significantly elevated at 14 and 28 days. Elastin is not a preferred substrate for plasmin,\textsuperscript{37} but MMPs do have elastinolytic activity.\textsuperscript{28,39} In these reinjured lesions, 95-kDa gelatinolytic activity (latent form of MMP-9) was detected at 1 to 2 days after the reinjury, but other gelatinolytic activities were not increased. We and others have not detected other elastases in the intima of rat arteries, and few macrophages, which possess an elastase, are detected in these lesions.\textsuperscript{10,24,40} Thus, an increase in tropoelastin expression is not accompanied by elastinolytic activity. Under these conditions, an increase in elastin would be anticipated. Indeed, electron microscopy confirms that elastin fibrils are abundant in the lesions of arteries after reinjury.

Why SMCs express tropoelastin in preference to other matrix proteins is not clear but may relate to a change in the ability of the artery to respond to the arterial wall stress. An increase in stretch and/or tensile stress is frequently associated with an increase in elastin synthesis by arterial SMCs.\textsuperscript{41,42} One possibility, therefore, is that the elastic lamellae were damaged by angioplasty and that as a reparative response, the SMCs express tropoelastin. This suggestion is supported by the finding that hypertension induces elastin expression and that increased stretch of pulmonary arteries causes a 9-fold increase in elastin with only a 2-fold increase in collagen.\textsuperscript{43} In rat arteries, Nikkari et al\textsuperscript{18} showed an increase in elastin as well as other matrix molecules after balloon injury, and the increase in expression of elastin was strong and remained high even after 28 days. Capron et al\textsuperscript{44} found a significant increase of elastin volume 14 days after reinjury to atherosclerotic rat aorta, although the same authors detected no significant increase in collagen volume. Furthermore, in a rabbit model, Strauss et al\textsuperscript{11} found that elastin content at 28 days increased \(\approx 30\%\) after the reinjury; however, collagen content was unchanged. Our observation of an increase in tropoelastin expression would support the concept that elastin is a major component of the arterial lesion after a sequential injury to an intimal lesion.

In the present study, we chose to examine those matrix proteins that are abundantly expressed in rat arteries. Furthermore, one hope was that we might provide collaborative data for at least \(\alpha_1(1)\)procollagen and tropoelastin from an ultrastructural examination of these arteries. With respect to tropoelastin, it would appear that our choice was well founded. These data, however, do not rule out the possibility that other matrix proteins such as proteoglycan(s) may contribute to the intimal lesion.\textsuperscript{11,18}

In a similar manner, other MMPs may be active in these arteries. We limited the present study to examine the gelatinases, since they are the only MMPs that we have found in both normal and injured rat arteries.\textsuperscript{24} Furthermore, it is possible that a change in the tissue inhibitors of MMP expression could influence MMP activity,\textsuperscript{45,46} and future studies will examine their role in these arteries.

TGF-\(\beta\) expression in the intima is upregulated after reinjury, and one consideration is that this cytokine is responsible for the increased expression of matrix proteins in these arteries, since there are many studies showing that TGF-\(\beta\) induces matrix synthesis in vascular cells.\textsuperscript{47-50} In the present study, TGF-\(\beta\) expression is upregulated for the entire 28 days after the reinjury, yet expression of \(\alpha_1(1)\)procollagen and fibronectin returned to control level at late times (14 to 28 days). One explanation for the weak correlation between expression of the matrix genes and TGF-\(\beta\)

The expression of proteases including TPA and MMPs has also been linked to the migration of SMCs into the intima.\textsuperscript{24,33,54} Indeed, a block of migration can impede the growth of intimal lesion in a balloon-injured artery.\textsuperscript{55,56} These data were obtained using rat arteries, which do not normally possess an intima with SMCs; therefore, migration of SMCs is necessary for the development of an intimal lesion. We do not know whether the movement of cells into the intima is important for the growth of lesions in arteries with a preexisting intima. In this present study, we were not able to directly measure the migration of cells, since this is not possible if an intima already exists. However, there was only a transitory increase in UPA activity with no change in either TPA activity or the gelatinolytic activities representing active forms of MMPs. This finding should be contrasted with the results following balloon catheter injury of a normal artery, after which increases in both TPA and MMP activity are detected for several days and there is a significant increase in cell migration.\textsuperscript{24,33,54} Thus, although there are no direct data, it would appear that migration of SMCs does not occur after angioplasty of a preexisting arterial lesion.

In summary, the present study showed that matrix accumulation was a critical reason for the lesion increase observed at 28 days after the reinjury and that the matrix accumulation depended on a balance between the matrix synthesis and degradation. Matrix synthesis was increased 7 days after the reinjury and partially continued up to 28 days, and the prolonged synthesis was mainly dependent on tropoelastin. Meanwhile, in matrix degradation, activity of UPA quickly increased after the reinjury and returned to the baseline level by day 14, whereas there was no change in TPA activity or
the gelatinolytic activities representing active forms of MMPs. These data explain why matrix accumulation appeared at 28 days after the injury and not at earlier time points.

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