Arterial Elastase Activity After Balloon Angioplasty and Effects of Elafin, an Elastase Inhibitor

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Abstract—Increased proteolytic activity may be a factor in intimal hyperplasia after balloon angioplasty (BA). The objectives of this study were to assess elastase activity after BA in a rabbit arterial double-injury model and the effects of elastase inhibition. Elastase activity increased immediately after BA, reached an 8-fold peak at 1 week, and declined to baseline levels by 4 weeks. Elastin zymography showed that the elastase activity was associated predominantly with a molecular mass of 25 kDa. Elastase activity was significantly inhibited in vitro by elafin and phenylmethylsulfonyl fluoride, selective inhibitors of serine elastases. A second group of animals was transfected after BA with a plasmid containing the cDNA for either elafin or a control (chloramphenicol acetyltransferase, CAT) construct by using a hemagglutinating virus of Japan–liposome transfection technique. Arterial segments were obtained at 48 hours, 1 week, and 4 weeks to assess transgene expression, arterial wall elastase activity, and intimal cross-sectional area, respectively. Elafin transgene expression was evident at 48 hours and resulted in a significant (80%) inhibition of elastase activity compared with chloramphenicol acetyltransferase–transfected arteries. There was a 43% reduction in intimal cross-sectional area in elafin-transfected arteries (0.28±0.22 versus 0.16±0.07 mm² for CAT-transfected versus elafin-transfected arteries, respectively; P<0.05). These data suggest that an early increase in serine elastase activity after BA contributes to intimal hyperplasia. Serine elastase inhibition may be a potential therapeutic approach to inhibit intimal hyperplasia. (Arterioscler Thromb Vasc Biol. 2001;21:1269-1274.)

Key Words: angioplasty ■ restenosis ■ elastase ■ elafin

The neointimal lesion that contributes to restenosis after balloon arterial injury is due to the migration of smooth muscle cells (SMCs) into the developing lesion and their production and the accumulation of extracellular matrix (ECM) proteins, mainly collagen and elastin, which make up the bulk of the intimal hyperplasia.1,2 There is increasing evidence that ECM degradative enzymes play an important role in SMC migration and ECM turnover, and increased elastolytic activity is implicated in the pathogenesis of several cardiovascular disease states involving neointimal formation,3 including pulmonary hypertension,4–6 transplant arteriopathy,7 and myocarditis.8 Inhibition of elastase activity in several animal models of pulmonary hypertension,9,10 transplant arteriopathy,11 and myocarditis8,12 has consistently resulted in the attenuation or reversal of pathology. Moreover, a transgenic mouse that overexpresses the serine elastase inhibitor elafin has a reduced neointima after carotid injury.13 All these studies suggest that increases in elastase activity may play an important causative role in the cardiovascular response to a variety of forms of injury.

Our group has previously demonstrated that after arterial balloon angioplasty (BA) injury, there is an early and marked increase in the turnover of the ECM proteins collagen and elastin that is followed by a later phase of ECM protein accumulation.1,2 This early elastin turnover suggests that BA injury upregulates elastolytic activity. Therefore, we sought to investigate the changes in elastolytic activity in response to BA injury and hypothesized that the inhibition of elastase in vivo would reduce the development of a neointima that contributes to restenosis.

Methods

Materials
cDNA encoding human elafin was obtained from Dr J.-M. Sallenave (University of Edinburgh, Edinburgh, Scotland). Hemagglutinating virus of Japan (HVJ) was generously provided by Dr Yasufumi Kaneda (Osaka University, Osaka, Japan). The following were purchased: HMG-1 protein (Wako Pure Chemicals), pcDNA expression vector (Invitrogen Corp), cholesterol and phosphatidylcholine (Sigma Chemical Co), phosphatidylyl serine (Avanti Polar Lipid), EnzChek elastase assay (Molecular Probes), elastin (No. KE57, Elastin Products Co), human elafin (Zeneca Pharmaceuticals), monoclonal antibodies against mouse neutrophils (MCA805 and MCA806), and Moloney murine leukemia virus reverse transcriptase and elafin and GAPDH primers (Canadian Life Technologies).
The Model
Animal experiments were carried out in accordance with guidelines set out by the University of Toronto and approved by the animal care committee at St. Michael’s Hospital. Normolipemic male New Zealand White rabbits, weighing 3.2 to 3.8 kg, were used. Time-course experiments for elastase activity and characterization of elastase inhibitors were performed in the iliac artery model, which we have previously used. Because carotid arteries are more accessible for intraluminal injections, gene transfer studies were performed in rabbit carotid arteries rather than the iliac arteries.

Iliac Artery Model
The double-injury iliac artery model was performed in 40 rabbits as previously described. The initial 4-cm segment of each iliac artery was injured by four 1-minute inflations with a BA catheter (3.0-mm diameter, 40-mm length) at 6, 8, 4, and 10 atm with 45 seconds between inflations. The iliac angioplasty procedure (injury 2) was repeated 3 weeks later. For the time-course study of elastolytic activity, animals were euthanized immediately after injury 2 at 1 week, at 4 weeks, or at 12 weeks. Injured arterial segments were harvested, immediately snap-frozen in liquid nitrogen, and maintained at −80°C until use. Uninjured iliac arteries served as controls.

Carotid Artery Model
To determine the effects of elastolytic activity on the formation of intimal hyperplasia after BA injury, we developed a strategy of inhibiting elastolytic activity by upregulating the expression of elafin, a serine elastase inhibitor, in the arterial wall after BA. These studies required transfection of the gene encoding for elafin at the time of the second BA injury (injury 2). Carotid arteries were injured using a BA catheter (3.5-mm diameter 30-mm length) with the same inflation protocol described above. Immediately after injury 2, the carotid arteries were exposed via a midline incision. The injured arterial segment was temporarily isolated with ligatures, and blood was flushed from the lumen by using a 30-gauge needle that was introduced into the arterial lumen proximal to the site of injury. A volume of 100 μL of the HVJ-liposome preparation was injected into the lumen to distend the arterial segment. The liposome solution (described below) was allowed to dwell for 20 minutes, and the arterial puncture was repaired. The protocol was repeated in the contralateral artery, and the neck incision was closed. Animals were euthanized at 3 time points: at 48 hours after injury 2 (n=5 rabbits) for transgene expression, at 7 days for elastase activity assay (n=12 rabbits), and at 4 weeks for measurement of intimal hyperplasia (n=15 rabbits). Arteries removed at 48 hours and 7 days were snap-frozen in liquid nitrogen and maintained at −80°C. The carotid arteries in animals euthanized at 4 weeks were pressure-fixed in situ with 10% formalin at 80 mm Hg for 20 minutes for morphometric analysis.

Preparation of HVJ-Liposomes
The cDNA encoding the amino acid sequence for human elafin was modified to produce a carboxyl terminal FLAG epitope−tagged fusion protein and ligated into the pcDNA3 expression vector containing a cytomegalovirus promoter, as previously described by our group. A control vector was prepared by using a cDNA encoding for chloramphenicol acetyltransferase (CAT) incorporated into the same plasmid. Bacteria were transformed, and plasmid DNA was purified and stored in 10 mmol/L Tris buffer, pH 8.5. HVJ-liposomes were prepared as described by Kaneda et al. Briefly, plasmid DNA was incubated with HMG-1 protein and then incorporated into liposomes formed with a lipid monolayer consisting of cholesterol, phosphatidylserine-sodium, and phosphatidylcholine. The resulting DNA/HMG-1-containing liposomes were then incubated with UV-inactivated HVJ and purified by sucrose density centrifugation. HVJ-liposomes were kept at 4°C and used within 48 hours.

Tissue Extraction
Frozen arteries were pulverized in liquid nitrogen and extracted in 0.5 mol/L sodium acetate buffer (pH 4.0) and 2.0 mmol/L methylamine for 30 minutes at 4°C. The sample was then centrifuged (1000g at 4°C) for 30 minutes, the supernatant was collected, and the pellet was extracted twice. Supernatants collected from 3 extraction steps were pooled and centrifuged (10 000g at 4°C) for 30 minutes. Supernatant was used as the source of elastase.

Cell Culture
Primary cultures of abdominal rabbit aortic SMCs were grown to ~80% confluence. Cultures were made quiescent in serum-free medium for 24 hours, after which the medium was replaced with fresh serum-free medium and incubated for 72 hours. Medium was collected and used as the source of elastase.

Partial Purification of Elastase
Solid ammonium sulfate (50% [wt/vol]) was dissolved into the tissue extracts or culture media and then brought at 4°C overnight to precipitate proteins. The samples were then centrifuged (10 000g at 4°C) for 30 minutes. Supernatant was discarded, and the pellet was dissolved in elastase assay buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L CaCl₂, 0.02% Brij 30, and 0.02% sodium azide, pH 7.6) and dialyzed (3500 MWCO Slide-A-Lyzer mini dialysis units) overnight against elastase assay buffer at 4°C. Protein concentration of the samples was measured by using a Bio-Rad protein assay kit.

Elastase Assays
Temporal changes in elastolytic activity in BA-injured iliac arteries were assessed by H-elastin degradation assay as previously described. Samples were added to H-elastin (100 000 cpm per assay) and incubated at 37°C for 18 hours and then centrifuged (8160g at 4°C) for 5 minutes. Radioactive solubilized elastin fragments were then measured with a scintillation counter.

After time-course experiments for elastase activity in injured arteries, all subsequent activity assays were performed by use of the EnzChek elastase assay kit. Elastase activity was measured at 37°C by using soluble bovine neck ligament elastin labeled with a fluorescent dye as a substrate. To identify the specific inhibitors for the elastase present in the arterial extracts or culture medium, the samples were incubated at room temperature with 10 mmol/L EDTA (matrix metalloproteinase inhibitor), 10 mmol/L phenylmethylsulfonyl fluoride (PMSF, serine elastase inhibitor), 1 mmol/L elafin (serine elastase inhibitor), or 10 mmol/L iodoacetamide (cysteine protease inhibitor) for 30 minutes before adding the substrate. Elastase activity is expressed as equivalent units of activity of porcine pancreatic elastase.

SDS-PAGE Zymography
To determine the molecular size of the elastase, arterial samples from uninjured and BA-injured (7 days after injury 2) arteries underwent elastase zymography in a 10% acrylamide gel containing 0.1% elastin. Samples from 9 arteries were pooled. Equal amounts of total protein were loaded onto SDS-polyacrylamide gel under nonreducing conditions without boiling. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 minutes and then incubated for 8 days at 37°C in elastase assay buffer. Lysis of the substrate in the gel was visualized by staining with Coomassie R-250 (0.5% [wt/vol]), followed by destaining overnight.

Detection of Exogenous Elafin mRNA by RT-PCR
RNA was extracted from a 2-cm segment of transfected carotid arteries by using Trizol reagent according to the manufacturer’s instructions. One microgram of the total RNA was subjected to reverse transcription (RT) by using Moloney murine leukemia virus reverse transcriptase and random primers in a total volume of 20 μL. Two microliters of the RT reaction mixture was used for the amplification of elafin and GAPDH in a polymerase chain reaction (PCR) with the use of 25 pmol human elafin or mouse GAPDH sense and antisense primers. The elafin primers were designed to generate a 150-bp fragment; the GAPDH primers generated a 343-bp fragment by PCR. The PCR procedure was carried out with an initial denaturation step of 30 seconds at 95°C, an annealing step of 45 seconds at 62°C, and 34 extension cycles at 72°C for 1 minute. The intensity of elafin bands was assessed visually relative to their respective GAPDH bands.
Marrow and spleen were used as positive controls. Human monocytes/macrophages (MAC387, 1:400). Rabbit bone also assessed at similar time points with an antibody directed against weeks after the second injury. Monocytes and macrophages were also examined for the presence of neutrophils at 1 week and 4 arterial cross sections from CAT- and elafin-transfected animals 72 hours after the second injury (4 vessels per time point). Carotid rabbits euthanized at 3 weeks after the first injury and at 24 hours and maximal intimal hyperplasia were used for statistical analysis.

Statistical Analysis
Data are expressed as mean±SD. To determine any temporal differences, a 1-way ANOVA was performed. If significant differences were found, the Tukey (Student method) range test was used to determine which time points were significantly different. In the gene transfection studies, unpaired t tests were used to compare the control (CAT) and treatment (elafin) groups. Statistical significance was defined as P<0.05.

Results

Temporal Changes in Elastolytic Activity
BA injury resulted in a marked transient increase in elastolytic activity as measured by ³H-elastin degradation (Figure 1). Changes in elastase activity appeared immediately after injury 2 and reached an 8-fold peak at 1 week after injury. Elastase activity levels returned to uninjured control levels by 4 weeks after injury.

Effects of Elastase Inhibitors
The arterial wall elastolytic activity was almost completely inhibited by selective inhibitors of serine elastases (Figure 2). Preincubation with PMSF or elafin resulted in 80% or 90% reduction in elastase activity, respectively. No inhibition was observed in samples incubated with the matrix metallopro-teinase (MMP) inhibitor, EDTA, or the cysteine protease inhibitor, iodoacetamide.

Zymography
In BA-injured arterial segments, the most prominent lytic band was present at a molecular mass of ≈25 kDa (Figure 3). A very faint lytic band was also apparent at 50 kDa. No lytic bands were evident in noninjured arteries.

SMC Elastase
Elastase activity produced by cultured SMCs was 0.084±0.021 mU/10⁶ cells per hour. Preincubation of samples with elafin or PMSF resulted in 97% or 84% inhibition of elastase activity, respectively. No significant effects (<5% inhibition) were observed with EDTA or iodoacetamide.

Immunohistochemistry
At 3 weeks after the first injury (ie, immediately before the second injury), only rare neutrophils (0 to 2 cells per cross section) were evident adherent to the endothelial layer (Figure 4A). No subintimal or adventitial neutrophils were found. The number of neutrophils after the second injury was related to the time point and extent of injury. At 24 hours, there were occasional neutrophils (2 to 4 cells per cross section) adherent...
to the deendothelialized luminal surface (Figure 4B). There were also few subintimal cells (0 to 4 cells per cross section), and in 1 case, there was a collection of \( \sim 30 \) neutrophils in a submedial location. Isolated adventitial neutrophils (2 to 25 cells per section) were also present. At dissection sites at 24 hours, there were prominent collections of neutrophils within the dissection plane as well as in overlying submedial and adventitial layers (Figure 4C). However, by 72 hours after the second injury, there was a dramatic reduction in neutrophils with only rare adherent neutrophils (0 to 2 cells per cross section) and adventitial neutrophils (0 to 4 cells per cross section). Even at dissection sites, only a few intact neutrophils were evident along with some cellular remnants (Figure 4D). Only rare monocytes/macrophages (0 to 1 cells per cross section) were present at any time point (data not shown).

**Elafin Transfection Studies**

At 48 hours after carotid artery injury 2 and gene transfection, a 150-bp elafin cDNA fragment was identified in elafin-transfected arteries but not in the injured CAT-transfected vessels or uninjured control vessels (Figure 5). At 7 days after BA injury, there was a significant decrease in elastase activity in the elafin-transfected arterial segments compared with the control (CAT-transfected) segments (0.50 \( \pm \) 0.14 versus 2.5 \( \pm \) 1.1 mU per artery per hour, \( P<0.05 \); Figure 6). At 4 weeks after BA injury, there was a 43% reduction in maximal intimal CSA (0.28 \( \pm \) 0.22 versus 0.16 \( \pm \) 0.07 mm\(^2\), \( P<0.05 \); Table) in elafin-transfected arteries (\( n=11 \)) compared with CAT-transfected arteries (\( n=7 \)). Similar differences in mean intimal CSA were also present. Total vessel area and lumen area were similar in the 2 groups (Table). No differences were found in SMC density between the 2 groups. Neutrophils were not evident in the intima of either group at 1 or 4 weeks.

**Discussion**

We have previously described the important role of ECM protein turnover in the development of the neointimal lesion, which results in restenosis.\(^1\) Furthermore, we have reported the presence of increased MMP activity in the arterial wall during the early period after BA injury.\(^2\) We now report a significant but transient increase in serine elastase activity in response to BA. The temporal profile of this phenomenon, with an early peak activity at 1 week followed by a return toward baseline levels at 4 weeks, is similar to that previously observed by us for the increase in elastin synthesis after BA injury. These results are consistent with the concept of an early period of intense proteolytic activity preceding the development of the bulk of the neointima.

A number of reports now support a key role for early proteolytic and, specifically, elastolytic activity in facilitating the proliferation and migration of SMCs and the subsequent accumulation of ECM. Several classes of elastolytic enzymes may contribute to the increased activity in response to injury, including MMPs, cysteine elastases, and serine elastases released by inflammatory cells, as well as endogenous elastase produced by SMCs in response to endothelial injury. MMPs with elastolytic activity are upregulated after balloon injury and are expressed by SMCs migrating into the developing neointima.\(^6\) Similarly, SMCs from atherosclerotic aorta express metalloelastases. Other investigators\(^17,18\) have noted that the inhibition of MMPs impairs SMC migration and transiently inhibits intimal hyperplasia. However, elastin accumulation is not prevented by the action of tissue inhibitor...
of metalloproteinase-1 in a rat model of restenosis.19 The potential role of non-MMP sources of elastase activity has received less attention, although Sukhova et al20 have found increases in the expression of cathepsins S and K, which are cysteine proteases that possess significant elastase activity, in neointimal macrophages and SMCs.

However, the failure of EDTA and iodoacetamide to significantly inhibit elastase activity 1 week after injury in the present study suggests that MMPs and cysteine proteinases do not contribute substantially to the elastase activity changes in our model. Indeed, the marked inhibition of arterial elastase activity with PMSF and elafin supports the presence of a serine elastase similar to that previously characterized in other models.6–8 The prominent lytic band on elastin zymography is also in keeping with the majority of the elastase activity being associated with a single enzyme, with only a small proportion of the activity due to other sources. The molecular mass of this band, 25 kDa, is similar to that for the endogenous vascular elastase that is increased in organ culture21 and in experimental models of pulmonary hypertension and coronary transplant arteriopathy.7 The elastase activity demonstrated in balloon-injured arteries and in SMC cultures suggests that an endogenous SMC elastase may also be involved in the development of the neointimal lesion in restenosis. However, neutrophil elastase has a similar molecular weight, and it is possible that the 25-kDa band represents an elastase expressed by leukocytes, which are present during the first week after injury. However, the marked reduction of neutrophil number in the vessel wall as early as 72 hours after the second injury with almost no detectable neutrophils evident at 1 week suggests that the increased and peak elastase activity present at 1 week is likely due to an SMC-derived elastase.

Regardless of the source of elastolytic activity, elastases could contribute to the development of neointima in a variety of ways.3 The disruption of the basement membrane and accelerated turnover of ECM would facilitate the migration of inflammatory cells and SMCs. Elastin products, the peptides of elastolytic activity, are chemotactic for leukocytes and induce an increase in fibronectin production by neointimal SMCs isolated from aorta or the ducxtus arteriosus.22 Fibronectin stimulates the development of a migratory SMC phenotype and could potentiate SMC recruitment into the ruptured plaque after BA. In addition, elastases may increase a number of growth factors that promote intimal hyperplasia. Latent forms of both transforming growth factor-β and basic fibroblast growth factor are bound to ECM proteins and are liberated by the action of elastolytic enzymes.23–25 Both of these growth factors have been shown to stimulate SMC proliferation and ECM synthesis. Finally, interleukin-8 and interleukin-1 are activated by elastases, further stimulating the inflammatory response to BA injury.26 Therefore, the increased elastolytic activity in response to BA injury may play an important role by stimulating a number of parallel processes, all of which contribute to restenosis.

The present study also showed that in vivo elafin overexpression after BA injury resulted in a significant (43%) reduction in intimal CSA compared with the control condition, further suggesting that serine elastases play a significant role in intimal hyperplasia formation. Elafin, an inhibitory protein normally expressed in skin, bronchia, and blood vessels, inhibits a number of serine elastases, including human leukocyte elastase and pancreatic elastase.27 It has been suggested that elafin plays a role in downregulating the inflammatory response in tissues commonly exposed to pathogens.28 Elafin has now been used successfully to prevent or reverse a number of cardiovascular disease states in which serine elastases have been shown to be involved in the pathogenesis, including myocarditis,8,1229 transplant coronary arteriopathy,13 and vein graft degeneration.14 The addition of the present finding of the effects of elafin in our model suggests that elafin may also be a useful therapeutic approach for conditions in which intimal hyperplasia plays a significant role, such as atrioventricular shunt stenosis and in-stent restenosis.

In summary, the present study suggests that balloon arterial injury in a rabbit model of restenosis results in increased activity of serine elastase activity early after BA injury and that elafin-transfected animals have decreased elastase activity associated with a decrease in intimal hyperplasia. These findings strongly support an important role for elastases in the pathogenesis of the neointimal lesion. The selective inhibition of elastase may, by preventing the activation of a variety of pathways, be a potential therapeutic target in the prevention of intimal hyperplasia and warrants further investigation.

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References


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