Differential Expression of Matrix Metalloproteinases After Stent Implantation and Balloon Angioplasty in the Hypercholesterolemic Rabbit

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Background—Intimal hyperplasia is the principal mechanism of in-stent restenosis. Matrix metalloproteinases (MMPs) play a key role in intimal growth after balloon angioplasty (BA). Little is known, however, about MMP expression after stent implantation (ST). We investigated whether MMP9 and MMP2 are differentially expressed after ST and BA.

Methods and Results—Hypercholesterolemic rabbits underwent ST and BA in the right and left iliac arteries, respectively. The expression of MMPs and their inhibitors (TIMPs) was studied at various time points in the injured arteries by use of zymography, reverse transcription–polymerase chain reaction, and immunohistochemistry. MMP2, but not MMP9, was constitutively expressed in uninjured arteries. MMP9 expression was rapidly induced after injury, whereas the increase in MMP2 expression was delayed. At all time points, pro-MMP9 activity and MMP9 mRNA levels were $2$-fold (ANOVA, $P=0.002$) and $3$-fold ($P$, $0.0001$) higher after ST than after BA, respectively. Active MMP9 was detected only after ST. Although the increases in MMP2 mRNA levels were of similar magnitudes after ST and BA, pro-MMP2 activity was slightly higher 7 and 30 days after ST, and MMP2 activity was $2$-fold higher 7 to 60 days after ST ($P=0.002$). No difference in TIMP expression was observed between stented and balloon-injured arteries. Cellular distributions of MMPs and TIMP1 were similar after ST and BA. Early inflammatory cell recruitment and 30-day intimal growth were more severe after ST.

Conclusions—Stent implantation results in more intense and sustained expression of MMP9 and activation of MMP2 than balloon angioplasty. (Circulation. 2001;103:3117-3122.)

Key Words: metalloproteinases ■ stents ■ angioplasty ■ restenosis
artery balloon angioplasty was performed with a 3-mm-diameter balloon (3X1-minute inflation, 10 atm); then a 15-mm-long Crown stent (Cordis) mounted over the balloon was implanted in the right iliac artery only (30-second inflation, 10 atm), as described. The animal protocol was approved by the Bichat University Institutional Animal Care and Use Committee. Animals were euthanized by pentobarbital overdose at 1 (n = 4), 3 (n = 4), 7 (n = 13), 30 (n = 4), and 60 (n = 3) days after injury. At each time point, right (stent) and left (balloon angioplasty) iliac arteries were harvested, flushed with ice-cold saline, cleaned of any adipose tissue, and divided into 2 or 3 segments. The proximal and distal segments were used for reverse transcription–polymerase chain reaction (RT-PCR) and gelatin zymography, respectively. In animals killed 1, 7, and 30 days after injury, an additional segment was taken from the center of the injury site and used for morphometric analyses and immunohistochemistry.

Unless indicated, stent struts were not removed before analysis so as to prevent the loss of MMP originating from adhering cells. The iliac arteries from 3 sham-operated (no arterial injury, hypercholesterolemic rabbits were used as negative controls. No significant variation in cholesterol levels was observed between baseline (620 ± 150 mg/dL) and death (610 ± 210, 595 ± 150, 605 ± 200, 625 ± 115, and 618 ± 175 mg/dL at 1, 7, 30, and 60 days, respectively).

Organ Culture and SDS-PAGE Zymography
Arterial segments were cut into rings 1 mm long and incubated for 24 hours in 1 mL serum-free DMEM (BioMedia) at 37°C in humidified 5% CO2/95% air. Preliminary experiments demonstrated that organ culture did not induce microscopically detectable tissue injury (data not shown). The conditioned medium was collected and stored at −80°C. Gelatinolytic activities of 20-μL samples of conditioned medium were measured as described. Results are expressed in densitometric units/mg wet wt. To verify the metalloproteinase nature of the detected enzymes, identical gels were expressed in densitometric units/mg wet wt. *P < 0.005; ‡P < 0.03; †P < 0.02; §P < 0.01. Organ culture did not induce microscopically detectable tissue injury (data not shown). The conditioned medium was collected and stored at −80°C. Gelatinolytic activities of 20-μL samples of conditioned medium were measured as described. Results are expressed in densitometric units/mg wet wt. To verify the metalloproteinase nature of the detected enzymes, identical gels were incubated in the presence of 30 mmol/L EDTA, an inhibitor of MMPs, or 1 mmol/L Pefabloc, a serine protease inhibitor.

Because binding of MMPs to their inhibitors (TIMPs) may have reduced MMP activity in conditioned media, samples were subjected to reduction in 2.5 mmol/L dithiothreitol and alkylation in 2.5 mmol/L iodoacetamide before electrophoresis. In addition, reverse zymography was performed to measure TIMP1 and TIMP2 inhibitory activities in conditioned media, as described.

Densitometric analyses of scanned gelatinolytic bands were performed with NIH Image 1.55 software.

Reverse Transcription–Polymerase Chain Reaction
Arterial segments were immediately frozen in liquid nitrogen and stored at −80°C. Procedures used for total RNA extraction and RT-PCR have been described. All RNA samples were run in parallel during the same PCR reaction, allowing for head-to-head comparisons between stented and balloon-injured arteries. The primers used to measure rabbit MMP9, MMP2, TIMP1, TIMP2, and GAPDH mRNA levels are listed in the Table. Results are expressed in arbitrary units and adjusted for GAPDH mRNA levels.

Morphometry and Immunohistochemistry
Stented and nonstented arterial segments were fixed in 4% paraformaldehyde and processed as described. Morphometric analyses were performed on 3 hematoxylin-phloxin-safran–stained cross sections for each artery.

Arterial specimens retrieved 7 days after injury were used for immunohistochemistry. Stent struts were gently removed with microforceps. Then arterial segments were embedded in OCT compound, frozen in liquid nitrogen–chilled isopentane, and stored at −80°C. Four-micrometer cross sections were obtained from each block and immunostained with mouse monoclonal antibodies (1:50 dilution, all from Oncogene unless indicated) directed against (1) MMP9 (Ab-1); (2) MMP2 (Ab-3); (3) TIMP1 (Ab-1); (4) TIMP2 (Ab-2); (5) RAM-11, a marker of rabbit macrophage cytoplasm (Dako); and (6) smooth muscle α-actin (HHF-35, Enzo Diagnostics), as described. For negative control experiments, primary antibodies were omitted.

### Statistical Analysis
Data are expressed as mean ± SD. MMP/TIMP activities and mRNA levels in stented versus balloon-injured arteries were compared by 2-way ANOVA (Statview 5.0, SAS Institute Inc), which tested the

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### Figure 1. MMP activities in injured arteries. A, Typical gelatinolytic activities in uninjured artery (sham) and 7 days after arterial injury. Pro-MMP9 (Ab-1); (2) MMP2 (Ab-3); (3) TIMP1 (Ab-1); (4) TIMP2 (Ab-2); (5) RAM-11, a marker of rabbit macrophage cytoplasm (Dako); and (6) smooth muscle α-actin (HHF-35, Enzo Diagnostics), as described. For negative control experiments, primary antibodies were omitted.

Data are expressed as mean ± SD. MMP/TIMP activities and mRNA levels in stented versus balloon-injured arteries were compared by 2-way ANOVA (Statview 5.0, SAS Institute Inc), which tested the
MMP Activities
In uninjured arteries, a single 70-kDa gelatinolytic band representing the latent form of MMP2 was observed (Figure 1).

Intense pro-MMP9 activity (98 kDa) was detected as soon as 1 day after arterial injury. At all time points, pro-MMP9 activity was ≥2-fold higher in stented arteries than in balloon-injured arteries (stent versus balloon, \( P < 0.002 \); time effect, \( P < 0.0001 \); interaction, \( P = 0.23 \)). Sixty days after stent implantation, pro-MMP9 activity was still ≥20-fold greater than control levels, whereas it was not different from control levels in balloon-injured arteries.

In 4 of 13 stented arteries studied 7 days after injury (but in none at other time points), a faint gelatinolytic band was observed at 80 kDa, which presumably represents the active form of MMP9.\(^{13}\) In no case was the active form of MMP9 detected in balloon-injured arteries.

There was no significant difference in pro-MMP2 activity between balloon-injured, stented, and uninjured arteries until day 7. At this time, pro-MMP2 activity increased in injured arteries, with slightly higher activities measured in stented arteries 7 and 30 days after injury (stent versus balloon, \( P = 0.006 \); time effect, \( P < 0.0001 \); interaction, \( P = 0.23 \)).

An additional gelatinolytic band at 60 kDa, representing the active form of MMP2,\(^{13}\) was observed 3 days after arterial injury. MMP2 activity peaked at day 30, then rapidly decreased. Seven to 60 days after arterial injury, MMP2 activity was ≥2-fold higher in stented than in balloon-injured arteries (stent versus balloon, \( P = 0.002 \); time effect, \( P < 0.0001 \); interaction, \( P = 0.02 \)). Sixty days after injury, MMP2 activity was still detectable in stented arteries but not in balloon-injured arteries.

All gelatinolytic activities were inhibited by EDTA but not by Pefabloc, suggesting that MMPs accounted for these activities (data not shown). Reduction-alkylation before gelatin zymography resulted in an ∼20% increase in pro-MMP9 activity, suggesting that part of pro-MMP9 was bound to its specific TIMP. This increase in pro-MMP9 activity, however, was similar in stented and balloon-injured arteries (data not shown). Reduction-alkylation had no effect on pro-MMP2 and MMP2 activities.

MMP Expression
Background MMP9 mRNA levels were measured in uninjured arteries. One day after injury, MMP9 mRNA levels increased dramatically, remained stable until day 30, then decreased (Figure 2). At each time point, MMP9 mRNA levels were ≥3-fold higher in stented arteries than in balloon-injured arteries (stent versus balloon, \( P < 0.0001 \); time effect, \( P = 0.0003 \); interaction, \( P = 0.2 \)). Sixty days after injury, high MMP9 mRNA levels were still present in stented arteries (∼5-fold greater than baseline) but not in balloon-injured arteries.

Constitutive MMP2 expression was found in uninjured arteries. MMP2 mRNA levels decreased slightly 1 day after injury, then increased significantly over time. Although MMP2 mRNA levels were slightly higher in stented arteries than in balloon-injured arteries between day 3 and day 30, no significant difference was observed (stent versus balloon, \( P = 0.3 \); time effect, \( P < 0.0001 \); interaction, \( P = 0.9 \)).

TIMP Inhibitory Activity and Expression
Both reverse zymography and RT-PCR demonstrated constitutive expressions of TIMP1 and TIMP2 (Figure 3). There was a nonsignificant increase of TIMP1 inhibitory activity after injury, with no difference between stented and balloon-injured arteries 7 and 30 days after injury (stent versus balloon, \( P = 0.002 \); time effect, \( P < 0.0005 \). C, Representative examples of MMP mRNA levels at various time points after stenting (ST) or balloon angioplasty (BA). Note that MMP9 mRNA is undetectable in uninjured artery (sham), which contrasts with constitutive expression of MMP2.
injured arteries (stent versus balloon, $P=0.3$; time effect, $P=0.06$; interaction, $P=0.2$). TIMP2 activity decreased significantly 1 day after injury and returned to baseline levels 30 days after injury. No difference in TIMP2 activity was observed between stented and balloon-injured arteries (stent versus balloon, $P=0.3$; time effect, $P=0.002$; interaction, $P=0.8$).

After arterial injury, both TIMP1 and TIMP2 mRNA levels increased transiently, with no difference between stented and balloon-injured arteries (TIMP1: stent versus balloon, $P=0.8$; time effect, $P=0.004$; interaction, $P=0.09$; TIMP2: stent versus balloon, $P=0.8$; time effect, $P<0.0001$; interaction, $P=0.7$).

Morphometry and Immunohistochemistry
The anatomy of the arterial wall appeared normal at baseline, i.e., after 14 days on the hyperlipidemic diet (data not shown). An intense recruitment of inflammatory cells with morphological features of polymorphonuclear leukocytes (PMNs) or monocytes was observed 1 day after injury in both stented and balloon-injured arteries (Figure 4). The number of adherent PMNs and monocytes was significantly higher after stenting $(74.5 \pm 21.1$ versus $21.1 \pm 6.0$) arteries. TIMP activities and mRNA levels are expressed in densitometric units (DU)/mg wet wt and in arbitrary units adjusted for GAPDH mRNA levels, respectively.

Figure 4. Pathology of stented and balloon-injured arteries. A and B, One day after arterial injury, recruitment of inflammatory cells (arrows) with morphological features of PMNs (A) or monocytes (B) is more intense in stented (A) than in balloon-injured (B) arteries. Magnification $\times 100$. Arrowheads indicate internal elastic lamina. C and D, Thirty days after injury, intimal hyperplasia is thicker in stented (C) than in balloon-injured (D) arteries. Note "bilayered" structure of intima (i) in stented arteries, with a thin, fibromuscular layer covering a large, foam cell–rich outermost layer. Magnification $\times 20$. m indicates media; a, adventitia; and *, stent strut.

MMP9, MMP2, and TIMP1 was similar in stented and balloon-injured arteries.

Immunostainings of adjacent cryosections revealed the constitutive expression of MMP2 and TIMP1 in medial SMCs of uninjured arteries (data not shown). MMP9 was found only in injured arteries and colocalized with intimal macrophages, intimal/medial SMCs, and adventitial fibroblasts (Figure 5). MMP2 was detected in intimal/medial SMCs and in adventitial fibroblasts. TIMP1 immunostaining was present in intimal/medial SMCs. TIMP2 was not detectable by immunohistochemistry. The cellular distribution of MMP9, MMP2, and TIMP1 was similar in stented and balloon-injured arteries.

Discussion
In the present study, we compared the impact of stent implantation and balloon angioplasty on the expression and activity of MMP9 and MMP2 in the iliac arteries of hypercholesterolemic rabbits. MMP9 expression was rapidly induced after arterial injury but more intensely and more durably after stent implantation. Stented arteries secreted higher levels of pro-MMP9 for $\geq 60$ days after injury. In addition, active MMP9 was detected only in stented arteries. In contrast, the increase in constitutive MMP2 expression was delayed and of similar magnitude in stented and balloon-injured arteries. The increase and, to a lesser extent, the production of pro-MMP2, however, were more pronounced in stented arteries. The expression of TIMPs did not differ between stented and balloon-injured arteries. The net increased proteolytic activity observed in stented arteries was associated with a more severe recruitment of inflammatory cells at day 1 and an enhanced neointimal growth at day 30 after arterial injury.

Previous studies performed in mice,$^{14}$ rats,$^{15-19}$ and rabbits$^{20-22}$ have demonstrated that arterial injury is a potent
activator of the MMP system. In the normocholesterolemic rabbit, balloon angioplasty of the iliac artery upregulates the transcription of the MMP2 gene and stimulates the transformation of pro-MMP2 into active MMP2. MMP9 activity, however, remains undetectable. In fact, in this model, only the combination of balloon injury and low flow results in detectable pro-MMP9 and MMP9 activities. In contrast, balloon injury combined with sustained atherogenic diet resulted in substantial pro-MMP9 activity, in addition to high MMP9 activity in both stented and balloon-injured arteries. MMP9 was detected in intimal macrophages (white arrows) and intimal/medial SMCs (black arrows), whereas MMP2 and TIMP1 were found in intimal/medial SMCs. Magnification ×100.

Our study brings new insight to the field of MMP response to arterial injury, in that we have included metallic stents, the principal technique used for percutaneous coronary interventions, in the design of our model. The main result of the present study is that overall, stents are more potent stimuli of MMP expression than balloon angioplasty. Given the key role of MMPs in cell migration and intimal growth and the recent findings that in-stent restenosis results almost exclusively from intimal hyperplasia, it is likely that MMPs are important contributors to in-stent restenosis. Indeed, we found that intimal area was ∼1.6-fold larger in stented arteries than in balloon-injured arteries.

Whether the more severe increase in MMP expression observed after stenting results from the recruitment of a larger population of MMP-expressing inflammatory cells or from a more pronounced upregulation of MMP expression in SMCs is difficult to determine. We were not able to fully investigate the cellular distribution of MMPs in stented arteries because, on the one hand, methyl methacrylate–embedded tissues do not lend themselves to standard immunohistochemistry protocols, and on the other hand, immunostaining of arterial cryosections requires that stent struts be retrieved before sectioning. This resulted in severe tissue damage (data not shown), precluding quantitative analysis of immunostained areas. Hence, no attempt was made to measure the specific contribution of each cell type to MMP9 and MMP2 activities. It is likely, however, that the more massive recruitment of PMNs observed 1 day after stent implantation participates in the early increase in MMP9 expression in stented arteries. Alternatively, MMP9-immunopositive SMCs were present in the media 7 days after arterial injury, whereas no MMP9 staining was detectable in uninjured arteries. Hence, MMP9 gene expression is induced in SMCs after arterial injury and may contribute to the higher MMP9 expression found in stented arteries.

We also provide evidence that MMP activation occurs after stenting. Activation of latent MMP9 was found only in stented arteries. However, it was inconstant (4 of 13 arteries), transient (≤7 days), and hardly quantifiable by zymography. In contrast, active MMP2 was detectable in both stented and balloon-injured arteries, but with much higher levels in stented arteries 7 to 60 days after injury.

Several mechanisms may be involved in the differential expression/activation patterns of MMPs in stented versus balloon-injured arteries. MMPs are regulated at 3 different levels: gene transcription, activation of latent pro-MMPs, and inactivation of MMPs by TIMPs. Proinflammatory cytokines—eg, interleukin-1—are key contributors to in-stent intimal hyperplasia and may be responsible, at least in part, for the induction of MMP expression in stented arteries. Recent studies suggest that reactive oxygen species or cell-cell or cell-matrix interactions may play a role as well.

Using both reduction-alkylation studies and direct measurements of TIMP expression, we provide evidence that the higher levels of MMP activities in stented arteries do not result from lower levels of TIMPs. Rather, the role of soluble activators of MMP9, such as plasmin, and the expression of MT1-MMP, a potent activator of pro-MMP2, deserve to be investigated.

Our study may have important implications for the understanding of the pathophysiology of in-stent restenosis and hence, the prevention of this phenomenon. Several authors have investigated the efficacy of MMP inhibitors on intimal growth after balloon injury. In rodents, synthetic MMP inhibitors only partially inhibited neointima formation, whereas overexpression of TIMPs, either via gene transfer or in genetically engineered mice, was more protective. N-Acetylcysteine has potent inhibitory effects on MMP9 in foam cells and deserves to be tested in vivo. On the basis of our findings that upregulation of MMP expression and MMP...
activation occur at much higher levels after stent implantation than after balloon angioplasty, the impact of MMP inhibitors on restenosis should be considered in stent models.

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References

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