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Overexpression of Urokinase by Plaque Macrophages Causes Histological Features of Plaque Rupture and Increases Vascular Matrix Metalloproteinase Activity in Aged Apolipoprotein E–Null Mice

Jie Hong Hu, PhD; Liang Du, MD; Talyn Chu, BS; Goro Otsuka, MD, PhD; Nagadhara Dronadula, PhD; Mia Jaffe; Sean E. Gill, PhD; William C. Parks, PhD; David A. Dichek, MD

Background—The mechanisms of atherosclerotic plaque rupture are poorly understood. Urokinase-type plasminogen activator (uPA) is expressed at elevated levels by macrophages in advanced human plaques. Patients with evidence of increased plasminogen activation have an elevated risk of major cardiovascular events. We used atherosclerotic mice to test the hypothesis that increased macrophage uPA expression in advanced plaques would cause histological features similar to those in ruptured human plaques.

Methods and Results—Bone marrow from transgenic mice with increased macrophage uPA expression or nontransgenic controls (all apolipoprotein E–null \[Apoe\]/H11002/H11002) was transplanted into 35-week-old Apoe/H11002/H11002 recipients, and innominate lesions and aortas were examined 8 to 13 weeks later. Donor macrophages accumulated in innominate lesions adjacent to plaque caps and in aortas, increasing uPA expression at both sites. Recipients of uPA-overexpressing macrophages had an increased prevalence of intraplaque hemorrhage (61% versus 13%; \(P=0.002\)) as well as increased lesion fibrin staining and fibrous cap disruption (\(P=0.06\) for both). Transplantation of uPA-overexpressing macrophages increased aortic matrix metalloproteinase activity (40%; \(P=0.02\)). This increase was independent of matrix metalloproteinase-9.

Conclusions—In advanced plaques of Apoe/H11002/H11002 mice, macrophage uPA overexpression causes intraplaque hemorrhage and fibrous cap disruption, features associated with human plaque rupture. uPA overexpression also increases vascular matrix metalloproteinase activity. These data provide a mechanism that connects macrophage uPA expression, matrix metalloproteinase activity, and plaque rupture features in mice. The data also suggest that elevated plaque plasminogen activator expression and plasminogen activation in humans may be causally linked to plaque rupture and cardiovascular events. (Circulation. 2010;121:1637-1644.)

Key Words: atherosclerosis • hemorrhage • metalloproteinases • plaque • urokinase

Atherosclerotic plaque rupture with intraplaque hemorrhage, intraluminal thrombosis, and vascular occlusion is the most common cause of myocardial infarction in humans.1 Nonocclusive plaque rupture is a likely cause of embolic stroke2 and may stimulate progression of arterial stenoses.3 Despite much work, the molecular and cellular mechanisms that cause plaque rupture remain poorly understood. Acquisition of a mechanistic understanding of plaque rupture could lead to novel diagnostic approaches that detect unstable plaques before they rupture and to the development of drugs that prevent plaque rupture.

Clinical Perspective on p 1644

Several causes of plaque rupture have been proposed, including the following: apoptosis of endothelial cells covering a plaque, exposing thrombogenic subendothelium4; apoptosis of smooth muscle cells in the plaque fibrous cap5,6 or heightened endoplasmic reticulum stress within plaque smooth muscle cells;7 both of which would weaken cap structure; rupture of plaque microvasculature, precipitating plaque hemorrhage8–10; rheological factors that alter plaque composition or otherwise precipitate thrombosis9,11; and increased plaque proteolytic activity that removes extracellular matrix, weakens plaque structure, and promotes smooth muscle cell apoptosis.6,12 Among these candidates, attention has focused primarily on increased proteolytic activity, most commonly attributed to matrix metalloproteinases (MMPs).5,13

A role for MMP-mediated proteolysis in plaque rupture is supported by correlative data demonstrating that various MMPs, which supposedly can digest key structural extracellular matrix components, are produced within vulnerable
regions of plaques. MMPs are translated as inactive proenzymes and require an activating protease to gain proteolytic function. Some MMPs are activated within the secretion pathway by proprotein convertase; however, for most MMPs, including essentially all MMPs implicated in plaque rupture, the activation mechanism is unknown. Plasmin, an abundant serine proteinase that could be activated within the plaque by macrophage-expressed urokinase plasminogen activator (uPA), is often considered to be a critical activator of MMPs in the vessel wall.

Despite wide acceptance of this model, it has been surprisingly difficult to obtain experimental data that support a causal role for either MMPs or uPA in plaque rupture. Studies in atherosclerosis-prone mice that overexpress or lack MMPs paint a complex and sometimes contradictory picture of the role of MMPs in lesion composition, with almost none of these studies showing an effect of MMPs on plaque rupture, defined as cap disruption with intraplaque hemorrhage. Two exceptions are as follows: a study in which adenosine expressing MMP-9 led to intraplaque hemorrhage in advanced carotid lesions of apolipoprotein E–null (Apoe–/–) mice and another study in which macrophage-targeted expression of a constitutively active MMP-9 mutant, but not wild-type MMP-9, led to intraplaque hemorrhage and cap disruption, also in Apoe–/– mice. Although these studies indicate that MMP-9 can cause histological features of plaque rupture, it remains unknown how endogenous proMMP-9 (or other proMMPs) would be activated within plaques. Similar studies in mice that either lack uPA or overexpress uPA in plaque macrophages have not yet revealed a role for this serine proteinase in plaque rupture, with 1 study suggesting that uPA contributes to plaque “stability.”

In the present study, we tested the hypothesis that increased expression of uPA in advanced atherosclerotic lesions will cause histological features of plaque rupture. To test this hypothesis, we introduced uPA-overexpressing macrophages into aged (35-week-old) Apoe–/– mice, documented accumulation of these macrophages in advanced atheroma of the innominate arteries and performed histological analyses of the plaques. We also tested whether increased expression of uPA in this model was associated with increased total MMP activity and, if so, whether MMP-9 activity was increased.

**Methods**

**Animals**

Apoe–/– mice with macrophage-targeted overexpression of uPA (SR-uPA+/– mice) were generated in our laboratory. The SR-uPA transgene includes a fragment of the human scavenger receptor (SR)-A promoter and the murine uPA gene. SR-uPA+/– mice were bred with nontransgenic Apoe–/– mice to yield SR-uPA+/0 or nontransgenic SR-uPA0/0 littermate controls. Some of the bone marrow (BM) donors used in the initial lineage-tracing study were also transgenic for green fluorescent protein (GFP). Because we modeled our study after that of Gough et al, we used a chow diet (catalog No. 5053, Labdiet). All animal protocols were approved by the University of Washington Office of Animal Welfare. See the online-only Data Supplement for further details.

**Experimental Design**

To test whether macrophage uPA overexpression induces histological features of plaque rupture in advanced atherosclerotic lesions, BM from either SR-uPA+/0 or nontransgenic male SR-uPA0/0 Apoe–/– donors was transplanted into lethally irradiated 35-week-old nontransgenic female Apoe–/– mice. Mice were killed 8 to 13 weeks after BM transplantation (BMT). BMT into older mice was necessary because germline transgenic SR-uPA+/0 Apoe–/– mice die at 15 to 30 weeks of age, before they can develop advanced atherosclerotic lesions.

**Bone Marrow Transplantation**

See the online-only Data Supplement.

**Plasma Lipids and Peripheral Blood Monocyte Counts**

See the online-only Data Supplement.

**Measurement of uPA mRNA and Activity**

Total RNA was extracted from innominate arteries. Arteries were harvested 10 weeks after transplant. In a subset of mice (8 per group), we excised aortic roots, emboled them in OCT, and sectioned them. See the online-only Data Supplement for further details.

**Tissue Harvest and Processing**

Innominate arteries were dissected free, placed in formalin for 48 hours, embedded in paraffin, and sectioned at the proximal end. Arteries examined for evidence of plaque rupture features were all harvested 10 weeks after transplant. In a subset of mice (8 per group), we excised aortic roots, emboled them in OCT, and sectioned them. See the online-only Data Supplement for further details.

**Histology and Lesion Characterization**

Entry of BM donor cells into recipient innominate lesions was investigated by staining serial innominate artery sections with antibodies to GFP (Molecular Probes, Carlsbad, Calif) and to the macrophage antigen Mac-3 (Pharmingen, San Diego, Calif). We analyzed innominate lesion histology using established methods. Stains in each set included the following: hematoxylin and eosin, picrosirius red to detect collagen, and Verhoeff–van Gieson to visualize medial elastin. Carstairs to detect fibrin and red blood cells, fibrinogen immunostain (Dako, Glodstrup, Denmark), and Mac-2 immunostain (Cedarlane Labs) to detect macrophages. Intraplaque hemorrhage, fibrin deposition, medial elastin breaks, and fibrous cap disruption were identified by an observer blinded to genotype, with the use of published criteria. These criteria included the following: Fibrinous cap disruption was present if at least 3 sections separated by 50 µm showed discontinuity or disruption of the fibrous cap accompanied by disruption of the underlying plaque structure. Intraplaque hemorrhage was defined as extraluminal red blood cells in at least 3 sections separated by 50 µm. Fibrin deposition was identified by the presence of both red-orange Carstairs stain and fibrinogen immunostain in the same location in a minimum of 3 step sections per artery. Intraplaque hemorrhages were assessed by a second blinded observer, whose assessments were nearly identical to those of the first observer. Sections of aortic roots were stained with hematoxylin and eosin and Mac-2, and intimal lesion area, intimal Mac-2–stained area, and percent Mac-2–stained area were measured. The presence of intraplaque hemorrhage was evaluated on hematoxylin and eosin–stained slides. See the online-only Data Supplement for further details.

**MMP Activity Assay**

Experimental samples, including aortic-conditioned media and human neutrophil lysates (obtained from normal human volunteers according to an approved institutional protocol), were incubated with OmniMMP Fluorogenic Substrate (Biomol) at 37°C in a black 96-well assay plate (Corning). Substrate cleavage was measured with a fluorometer (BioTek) with the use of an excitation filter of 328 nm and emission filter of 393 nm.

**Gelatin Zymography**

Aortic explant culture media, aortic extracts, and neutrophil lysate were separated under nondenaturing conditions by 10% SDS-PAGE in gels containing 0.5 mg/mL gelatin. Neutrophil lysate was used as a...
positive control for MMP-9 gelatinolytic activity. See the online-only Data Supplement for further details.

Immunoprecipitation and Western Blotting
MMP-9 in aortic-conditioned media and neutrophil lysate was removed and identified by immunoprecipitation and Western blotting with the use of protein G Sepharose beads (Invitrogen) and an antibody to rat MMP-9 that also binds human and mouse MMP-9 (Millipore No. AB19016; 1:100 dilution) or an antibody to GAPDH (Sigma, St Louis, Mo). See the online-only Data Supplement for further details.

Statistical Analysis
Data are mean±SD or median (25% to 75% range). A priori hypotheses were tested with the unpaired t test or by Mann-Whitney rank sum test when group data were nonnormally distributed or if group variances were unequal. Survival rate curves were generated with the Kaplan-Meier method and compared with the log-rank test. The Fisher exact test was used to compare the prevalence of intraplaque hemorrhage, fibrin deposition, and fibrous cap disruption.

Results
Hematopoietic Reconstitution and Population of Atherosclerotic Lesions by Donor-Derived Macrophages
Flow cytometry of peripheral blood of recipients of GFP+/0 BM, performed 12 to 13 weeks after BMT, showed successful reconstitution (83±5% GFP+ cells; n=7; data not shown). Immunohistochemical staining of innominate arteries of GFP+/0 BM recipients revealed abundant, foamy intimal cells that expressed both GFP and Mac-3 (Figure 1).

uPA Expression in Innominate Arteries and Aortas of SR-uPA+/0 BM Recipients
uPA mRNA was elevated in innominate arteries of recipients of SR-uPA+/0 BM (2.2 [2.1 to 2.3] versus 0.03 [0.02 to 0.08] arbitrary units in recipients of nontransgenic BM; P=0.001; Figure 2A). PA activity in medium conditioned by aortas of recipients of SR-uPA+/0 BM was similarly increased (1.5 [1.3 to 1.6] versus 0.04 [0.03 to 0.09] IU/mg protein in medium conditioned by aortas from recipients of nontransgenic BM; P=0.001; Figure 2B).

Peripheral Blood Monocytes, Plasma Cholesterol, and Body Weights
Engraftment of SR-uPA+/0 BM did not affect the total number or percentage of peripheral blood monocytes (P=0.3 for both; Table 1). Recipients of SR-uPA+/0 BM had modestly higher plasma total cholesterol (38%) and slightly lower body weights (8%) than recipients of nontransgenic BM (P=0.01 for both; Table 1).

Figure 1. Colocalization of GFP and Mac-3 expression in an innominate artery plaque. The artery was removed 12 to 13 weeks after BMT of GFP+/0 BM. A, Immunostain for GFP, to detect cells from BM donor. B, Adjacent section immunostained for Mac-3 (a macrophage antigen). C and D, Sections stained with primary antibody controls for GFP and Mac-3, respectively. Bar=100 μm. L indicates lumen; arrows, fibrous cap.

Figure 2. Elevated uPA expression in innominate arteries and aortas from recipients of SR-uPA+/0 BM. A, uPA mRNA measured by quantitative reverse transcriptase-mediated polymerase chain reaction and normalized to GAPDH mRNA. AU indicates arbitrary units. B, Plasminogen activator (PA) activity in medium conditioned by explanted aortas. Innominates and aortas are from recipients of nontransgenic (SR-uPA0/0) or transgenic (SR-uPA+/0) BM. Data points are individual mice; bars are group medians, compared by Mann-Whitney test.
Sudden Death–Free Survival After BMT
Recipients of SR-uPA^+/0^ BM began to die suddenly starting 6 weeks after BMT. By 10 weeks after BMT, 32% of the SR-uPA^+/0^ recipients had died suddenly compared with 9% of the recipients of nontransgenic BM (P=0.001; Figure I in the online-only Data Supplement). The sudden death phenotype is also present in SR-uPA^+/0^Apoe^−/−^ mice and younger Apoe^−/−^ recipients of SR-uPA^+/0^ BM.16-18 The premature deaths of younger mice with SR-uPA^+/0^ BM appear to be due to occlusive coronary atherosclerosis without plaque rupture (no ruptured aortic root or coronary artery plaques were seen in younger mice)23,32; however, the proximate cause of death in these mice and in the older mice in the present study (including possible arrhythmias, thromboembolism, or acute pump failure) remains unclear.

Innominate Plaque Volume, Vessel Size, Lesion Composition, and Elastin Destruction
Innominate artery intimal, medial, lumen, and total vessel areas did not differ between recipients of SR-uPA^+/0^ and recipients of nontransgenic BM (P=0.3 for all except lumen area, which was slightly [15%] smaller in SR-uPA^+/0^ mice with borderline significance; P=0.08; Table I in the online-only Data Supplement). There were also no differences between the groups in lesion collagen area and macrophage content (either total or percentage; Table 2 and Figure IIB and IIC in the online-only Data Supplement) or in the number of elastin breaks in the underlying media (Table 2 and Figures IIF and III in the online-only Data Supplement).

Histological Features of Plaque Rupture
Innominate intraplaque hemorrhage was significantly more prevalent in recipients of SR-uPA^+/0^ BM (11 of 18 [61%] in SR-uPA^+/0^ recipients versus 3 of 23 [13%] in recipients of nontransgenic BM; P=0.002; Figure 3A, 3B, 3C, and 3F and Table 2). Because neither we nor several other groups that have reported murine plaque hemorrhage have observed microvessels in innominate plaques of Apoe^−/−^ mice,33 we attribute plaque hemorrhage to entry of luminal blood through a disrupted plaque cap. Fibrin accumulation (Figure IID and IIE in the online-only Data Supplement) and fibrous cap disruption (Figure 3D and 3E) were also increased in recipients of SR-uPA^+/0^ BM, although statistical significance was borderline (P=0.06 for both; Table 2). Macrophages were always present at sites of fibrous cap disruption (Figure 3D and 3E).

Histological Analysis of Aortic Root Lesions
Intimas of aortic root lesions were not significantly larger in recipients of SR-uPA^+/0^ BM (5.9±0.38 versus 4.9±0.54 μm^2^×10^3^; P=0.2). However, aortic root intimas of recipients of SR-uPA^+/0^ BM had a significantly higher macrophage content (0.62±0.056 versus 0.37±0.060 μm^2^×10^3^; P=0.01) and a higher percentage of Mac-2–stained area (11±0.85% versus 7.4±0.75%; P=0.01) than were present in recipients of nontransgenic BM. Intraplaque hemorrhage was present in 2 of 8 aortic root lesions in recipients of SR-uPA^+/0^ BM and 1 of 8 lesions in recipients of nontransgenic BM (P=1.0). These hemorrhages were small, with far fewer extravasated red cells than in the innommates.

Aortic Total MMP Activity
Because innominate arteries are too small to generate sufficient conditioned media for the MMP activity assay, we used aortic-conditioned media to investigate whether transplantation of SR-uPA^+/0^ macrophages increased vascular MMP activity. Total MMP activity was 40% higher in medium conditioned by aortas of SR-uPA^+/0^ BM recipients (18±4.0 versus 13±2.9 Δ relative fluorescent units per min per microgram protein for aortic-conditioned medium from SR-uPA^+/0^ BM recipients; P=0.02; Figure 4). Control experiments showed that neither uPA, uPA with added plasminogen, nor plasmin cleaved the MMP substrate (data not shown).

Table 1. Systemic Effects of SR-uPA Transgene in BM Recipients

<table>
<thead>
<tr>
<th>BM Donor Genotype</th>
<th>SR-uPA^+/0^</th>
<th>n</th>
<th>SR-uPA^+/0^</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripher al blood monocytes, cells/mm^3^</td>
<td>150±120</td>
<td>7</td>
<td>87±68</td>
<td>6</td>
<td>0.3</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td>2.3±1.4</td>
<td>7</td>
<td>3.2±1.9</td>
<td>6</td>
<td>0.4</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dL</td>
<td>330±160</td>
<td>22</td>
<td>460±130</td>
<td>17</td>
<td>0.01</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>25±1.8</td>
<td>23</td>
<td>23±1.9</td>
<td>18</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Values are reported as mean±SD, compared by t test. n indicates number of recipient mice.

Table 2. Innominate Artery Lesion Content and Histological Features

<table>
<thead>
<tr>
<th>BM Donor Genotype</th>
<th>SR-uPA^+/0^</th>
<th>n</th>
<th>SR-uPA^+/0^</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion collagen area, ×10^-3^ mm^2^</td>
<td>26 (18–45)</td>
<td>22</td>
<td>21 (18–42)</td>
<td>17</td>
<td>0.8</td>
</tr>
<tr>
<td>Collagen content, % total lesion</td>
<td>20 (14–28)</td>
<td>22</td>
<td>19 (14–28)</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Lesion Mac-2–positive area, ×10^-3^ mm^2^</td>
<td>8.6 (5.6–17)</td>
<td>22</td>
<td>9.1 (6.6–14)</td>
<td>18</td>
<td>0.6</td>
</tr>
<tr>
<td>Macrophage content, % total lesion</td>
<td>7.6 (4.6–12)</td>
<td>22</td>
<td>6.5 (4.1–12)</td>
<td>18</td>
<td>0.9</td>
</tr>
<tr>
<td>Intraplaque hemorrhage</td>
<td>3/23 (13)</td>
<td>23</td>
<td>11/18 (61)</td>
<td>18</td>
<td>0.002</td>
</tr>
<tr>
<td>Fibrin deposition</td>
<td>8/23 (35)</td>
<td>23</td>
<td>12/18 (67)</td>
<td>18</td>
<td>0.06</td>
</tr>
<tr>
<td>Medial elastin breaks (per section)</td>
<td>0.50 (0.25–1.8)</td>
<td>21</td>
<td>0.56 (0.25–1.4)</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Fibrous cap disruption</td>
<td>11/23 (48)</td>
<td>23</td>
<td>14/18 (78)</td>
<td>18</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Continuous data are reported as median (25% to 75%) range and are compared by Mann-Whitney test. Categorical data are reported as mice with indicated feature/total mice in the group (%) and are compared by Fisher exact test. n indicates number of recipient mice.
Gelatin zymography of aortic-conditioned media and aortic extracts of BMT recipients revealed variable levels of active MMP-9 with no consistent difference between the groups (Figure 5A and data not shown). To assess secreted MMP-9 activity directly, we immunoprecipitated the enzyme from aortic-conditioned media and quantified the activity recovered. Immunoprecipitation of MMP-9 removed all MMP activity from MMP-9–rich neutrophil lysate (Figure IV in the online-only Data Supplement) and also removed substantial immunoreactive MMP-9 from aortic-conditioned media (Figure 5B). However, immunoprecipitation of MMP-9 from aortic-conditioned media did not alter total MMP activity (Figure 5C).

Discussion

We used a BMT model and older Apoe\(^{-/-}\) mice to investigate whether macrophage uPA overexpression in advanced atherosclerotic lesions induces histological features of plaque rupture. Our major findings were as follows: (1) Donor BM-derived macrophages entered mature innominate lesions and became foam cells; (2) recipients of SR-uPA\(^{+/0}\) BM expressed significantly higher levels of uPA in innominate artery and aorta; (3) recipients of SR-uPA\(^{+/0}\) BM had a higher prevalence of intraplaque hemorrhage, plaque fibrin accumulation, and fibrous cap disruption; (4) MMP activity was elevated in aortas of recipients of SR-uPA\(^{+/0}\) BM; and (5) MMP-9 does not contribute to the increase in aortic MMP activity. These data support a causal role for macrophage uPA overexpression in atherosclerotic plaque rupture and suggest that macrophage uPA overexpression induces plaque rupture by activation of MMP(s) other than MMP-9.

One of the key assumptions of this study is that the hyperlipidemic mouse innominate artery is an informative experimental setting for elucidating the molecular and cellu-
of all murine models of human atherosclerosis,37 the mouse have increased expression of plasminogen activators and ele-
ments of nontransgenic (SR-uPA<sup>0/0</sup>) or transgenic (SR-uPA<sup>+/+</sup>) BM was analyzed by gelatin zymography (A), immunoprecipitation and Western blotting (B), and MMP activity assay (C). A. Samples are from individual mice; “PMN control” is human neutrophil lysate (a positive control). B. MMP-9 Western blot of eluate of Sepharose beads used to immunoprecipitate aortic-conditioned medium. Beads were linked to antibodies to either MMP-9 or GAPDH. C. Total MMP activity in aortic-conditioned medium, measured after immunoprecipitation with antibodies to either MMP-9 or GAPDH. Data points are individual mice; bars are group means.

Figure 5. MMP-9 activity and antigen in aortic-conditioned medium. Medium conditioned by aortas explanted from recipients of nontransgenic (SR-uPA<sup>0/0</sup>) or transgenic (SR-uPA<sup>+/+</sup>) BM was analyzed by gelatin zymography (A), immunoprecipitation and Western blotting (B), and MMP activity assay (C). A. Samples are from individual mice; “PMN control” is human neutrophil lysate (a positive control). B. MMP-9 Western blot of eluate of Sepharose beads used to immunoprecipitate aortic-conditioned medium. Beads were linked to antibodies to either MMP-9 or GAPDH. C. Total MMP activity in aortic-conditioned medium, measured after immunoprecipitation with antibodies to either MMP-9 or GAPDH. Data points are individual mice; bars are group means.

lar pathophysiology of human plaque rupture. The mouse innominate model was initially widely embraced because, unlike other animal models of atherosclerosis, it exhibits fibrous cap disruption and intraplaque hemorrhage, 2 key histological features of human plaque rupture.26,34 Although this model remains widely used,22,35,36 it has also been criticized because it does not replicate other well-described histological features of human plaque rupture including occlusive thrombi, eroded endothelium, and luminal eruption of calcific nodules.33 Despite these limitations, akin to those of all murine models of human atherosclerosis,22 the mouse innominate is currently the best available animal model for unraveling the molecular and cellular events that lead to human plaque rupture.

Our finding that BMT of uPA-overexpressing macrophages and their accumulation in plaque shoulder areas led to fibrous cap disruption and intraplaque hemorrhage (Figure 3 and Table 2) provides a mechanistic connection between histological data examining plasminogen activation in human plaques and data from clinical studies. uPA is highly expressed in macrophage-rich areas adjacent to the shoulder regions of advanced human atherosclerotic lesions.16,17,38 “Culprit” human carotid plaques have increased expression of plasminogen activators and elevated plasmin activity compared with uncomplicated plaques,39 and both elevated plasminogen activator mRNA and increased uPA receptor expression by macrophages correlate with intraplaque hemorrhage and rupture in acutely symptomatic human carotid plaques.40,41 In the clinical realm, low plasma high-density lipoprotein was associated with elevated plasminogen activation by human peripheral blood monocytes (likely due to upregulation of monocyte uPA receptors).42 a small study revealed elevated plasma uPA in patients with plaque rupture visualized by intravascular ultrasound,43 and 3 large clinical trials associated increased plasminogen activation (measured as plasma plasmin-antiplasmin complexes) with risk of major cardiovascular events.44–46 The present study, by linking plaque plasminogen activation and histological features of plaque rupture, connects these histological and clinical observations.

Fitting the results of the present study with data from other studies in which uPA expression was manipulated in atherosclerotic mice is more of a challenge. Nevertheless, in all cases apparent discrepancies can be explained. In an early study of atherosclerosis in uPA knockout mice, no effects of uPA deficiency on cap disruption and intraplaque hemorrhage were reported;46 however, only aortic root lesions were examined, and neither cap disruption nor intraplaque hemorrhage is typically found in the mouse aortic root. In a more recent study of older, atherosclerotic uPA-null mice, inominate lesions were examined, but neither cap disruption nor intraplaque hemorrhage was among the end points reported and might have been overlooked.25 In our own work, both SR-uPA<sup>+/+</sup> transgenic mice and younger recipients of SR-uPA<sup>+/+</sup> BM had accelerated aortic atherosclerosis, whereas in the present study we found no effect of uPA overexpression on inominate or aortic root plaque volume (Table I in the online-only Data Supplement and Results, above). This apparent discrepancy may indicate a stage-specific role for uPA in lesion growth, with little effect of uPA on growth of advanced lesions in these 35- to 45-week-old mice.26

Our study provides biochemical data that support a stepwise connection between increased plasminogen activation, MMP activation, and plaque rupture. These connections have long been hypothesized but heretofore lacked experimental support. A recent report established an association between plasmin, MMP-9, and experimental murine aortic aneurysms.31 However, despite numerous experiments in atherosclerotic MMP-knockout and MMP-overexpressing mice, only scant data support the long-hypothesized role of MMPs in plaque rupture. The study that most convincingly links MMP activity and mural plaque rupture relied on intraplaque expression of a constitutively active MMP-9 mutant, leaving open the question of why, in the same study, overexpression of wild-type, activatable MMP-9 in rupture-prone inominate plaques did not cause plaque rupture.22 Here we provide a possible answer to this conundrum by showing that overexpression of wild-type, activatable uPA increases MMP activity significantly and precipitates plaque rupture via an MMP-9-independent pathway. Thus, overexpression of active MMP-9 might cause plaque rupture experimentally, but the native pathway through which plaques rupture may be via uPA, plasmin, and a different MMP. Future work will be aimed at identifying the MMP(s) activated by uPA in this model and connecting the activity of these MMP(s) with cap disruption and intraplaque hemorrhage. Finally, more widespread use of the highly quantitative total MMP activity assay (Figure 4) would facilitate comparisons among studies of vascular MMP overexpression, poten-
tially clarifying why plaque rupture features are found in some studies but not in others.20

Our study has limitations. First, the 30-fold overexpression of uPA in mouse innominate and aortas (Figure 2) is higher than the level of uPA overexpression we achieved in aortas of younger mice with macrophage-targeted uPA overexpression23,32 and could be viewed as nonphysiological. It would be interesting and valuable to test whether lower levels of uPA overexpression in mature plaques, over longer periods of time, also caused plaque rupture, but this is not possible with the present model. Second, in contrast to our previous studies in which the SR-uPA transgene did not affect plasma cholesterol,23,24,32 we also observed a modest (40%) increase in plasma cholesterol in recipients of SR-uPA40 BM. However, plasma cholesterol levels of mice with histological features of plaque rupture did not differ significantly from cholesterol levels of mice without plaque rupture (data not shown), and the increased plasma cholesterol in SR-uPA40 BM recipients did not affect innominate lesion volume (Table I in the online-only Data Supplement). Both of these observations argue that differences in plasma cholesterol between the groups were not responsible for the increased intraplaque hemorrhage.

In summary, our data support a causal connection between elevated lesion uPA expression, increased vascular MMP activity, disruption of plaque structural integrity, and intraplaque hemorrhage. The clinical relevance of these data, generated in an animal model, is supported by human pathological and clinical studies that associate increased plasminogen activation with unstable plaques and major cardiovascular events. Future studies in this model might identify key substrates (such as matrix molecules) whose cleavage leads to plaque rupture. We also speculate that clinical strategies aimed at limiting vascular wall plasminogen activation might inhibit plaque rupture, thereby preventing both myocardial infarctions and strokes.

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Disclosures

None.

References


37. Rader DJ, Daugherty A. CLINICAL PERSPECTIVE

Rupture of previously stable atherosclerotic plaques causes unstable angina, myocardial infarctions, and strokes. However, the mechanisms that cause plaque rupture are not yet understood. If these mechanisms could be identified, therapies might be developed that prevent plaque rupture. The most widely held mechanistic hypothesis relative to plaque rupture is that it is caused by increased activity of plaque proteolytic enzymes including urokinase, plasmin, and matrix metalloproteinases. This hypothesis is supported by histological analyses of human plaques that show active proteases in ruptured plaques and by clinical studies that associate higher levels of proteolytic activity (in plasma) with major cardiovascular events. However, these studies do not prove causality, and a large number of animal studies have not revealed a reliable cause-and-effect relationship between plaque proteolytic activity and histological features of plaque rupture. Here we generated a new animal model in which we introduce macrophages that express high levels of urokinase into advanced atherosclerotic plaques in mice. Arteries from these mice have elevated proteolytic activity, including high levels of both plasminogen activator and matrix metalloproteinase activity. Compared with control mice, mice with urokinase-type plasminogen activator–overexpressing macrophages have a significantly higher (61% versus 13%) prevalence of intraplaque hemorrhage as well as a higher prevalence of plaque cap disruption and fibrin(ogen) staining (features found in ruptured human plaques). These data provide experimental support for a mechanistic connection between macrophage urokinase-type plasminogen activator expression, matrix metalloproteinase activity, and plaque rupture. These data also support a causal link between elevated plaque plasminogen activator expression, plaque rupture, and cardiovascular events in humans.
SUPPLEMENTAL MATERIAL

Expanded Methods

Animals

$\text{Apoe}^{-/-}$ mice with macrophage-targeted overexpression of uPA (SR-uPA $^{+/0}$ mice) were generated in our laboratory. The SR-uPA transgene includes a fragment of the human scavenger receptor (SR)-A promoter and the murine uPA gene. SR-uPA $^{+/0}$ $\text{Apoe}^{-/-}$ mice were bred with non-transgenic $\text{Apoe}^{-/-}$ mice to yield SR-uPA $^{+/0}$ or non-transgenic SR-uPA $^{0/0}$ $\text{Apoe}^{-/-}$ littermate controls. Male mice were used as bone marrow (BM) donors. Some of the donors used in the initial lineage-tracing study were also transgenic for green fluorescent protein (GFP). Female $\text{Apoe}^{-/-}$ recipient mice were bred or purchased (Jackson Laboratory). All experimental mice in this study were progeny of C57BL/6 backcrosses for at least 10 generations. Because we modeled our study after that of Gough et al., we fed the mice a chow diet (Catalog # 5053, Labdiet). Mice were housed in a specific-pathogen-free facility, and genotyped by PCR for the SR-uPA and GFP alleles. All animal protocols were approved by the University of Washington Office of Animal Welfare.

Experimental Design

To test if macrophage uPA overexpression induces histologic features of plaque rupture in advanced atherosclerotic lesions, BM from either SR-uPA $^{+/0}$ or non-transgenic SR-uPA $^{0/0}$ $\text{Apoe}^{-/-}$ donors was transplanted into lethally irradiated 35-wk old non-transgenic $\text{Apoe}^{-/-}$ mice. Mice were killed 8–13 wk after BM transplant (BMT). BMT into older mice was necessary because germline transgenic SR-uPA $^{+/0}$ $\text{Apoe}^{-/-}$ mice die at 15–30 wk of age, before they can develop advanced atherosclerotic lesions.

Bone Marrow Transplantation (BMT)
BM recipients received neomycin water (2 mg/ml) for 1 wk before and 2 wk after BMT. Recipients were irradiated with 10.5 Gy (Cesium-137 γ-ray source) and injected via tail vein with approximately 1.2 x 10^7 BM cells from 7 – 12-wk-old donors. BM reconstitution was assessed by flow cytometry of peripheral blood 12 – 13 wk after BMT. Blood was lysed with 1X red blood cell lysis buffer (15.5 mM NH₄Cl, 1.0 mM KHCO₃, 0.1 mM EDTA) for 2 min at room temperature, washed with ice cold-PBS with 2% FBS and heparin (5 U/mL), suspended with ice cold-PBS with 2% FBS, stained with Propidium Iodide (PI, 1 μg/mL, to identify dead cells) and analyzed for GFP expression. Peripheral blood cells from GFP⁺/0 and GFP⁰/⁰ mice were positive and negative controls.

**Plasma Lipids and Peripheral Blood Monocyte Counts**

Total cholesterol was measured in blood obtained from the retro-orbital plexus of BM recipients, 8 – 11 wk post-transplant (Spectrum cholesterol assay, Abbott). Complete blood counts were performed by an outside laboratory (Phoenix Central Laboratory).

**Measurement of uPA mRNA and Activity**

8 – 11 wk after BMT, recipients were deeply anesthetized and perfused for 5 min with 0.9% saline. Innominate arteries were dissected out and snap-frozen in liquid N₂. Aortae were removed, trimmed, rinsed, and incubated overnight in M199 (GIBCO). Conditioned media and aortae were stored at -80 °C. Total RNA was extracted from innominate arteries using the RNeasy Kit (Qiagen) and quantified by Nanodrop (Thermo Scientific). RNA quality was verified by agarose gel electrophoresis and ethidium bromide staining. uPA and GAPDH mRNA were measured by qRT-PCR (TaqMan® Rodent GAPDH Control Reagents; Applied Biosystems), as described.

Plasminogen activator (PA) activity in aortic-conditioned medium was measured by incubating medium with Glu-plasminogen and S-2251. PA activity was calculated with reference to human single-chain uPA (American Diagnostica), and normalized to total protein.
Control experiments verified that S-2251 cleavage in this assay is plasminogen-dependent.\(^5\)

**Tissue Harvest and Processing**

At 10 – 13 wk post-transplant, mice were deeply anesthetized and perfused via the left ventricle for 5 min with 0.9% saline, and for 5 min with 10% phosphate-buffered formalin pH 7.4, containing 220 mM sucrose, 2 mM EDTA, 0.02 mM BHT (Sigma). Innominate arteries were dissected free from the aortic arch to the bifurcation. Close attention was paid to avoid applying traction to the innominates, which were never touched directly. Excised arteries were placed in formalin for over 48 h, embedded in paraffin, and sectioned starting at the proximal end. Arteries examined for evidence of plaque rupture features were all harvested 10 wk post-transplant. In a subset of mice (8 per group) we excised aortic roots, embedded them in OCT, and sectioned them.\(^1\)

**Histology and Lesion Characterization**

Entry of BM donor cells into recipient innominate lesions was investigated by staining serial innominate artery sections with antibodies to GFP (Molecular Probes) and to the macrophage antigen Mac-3 (PharMingen). Rabbit IgG (Dako) and Rat IgG1κ (PharMingen) were used as primary antibody controls, respectively. We analyzed innominate lesion histology using established methods.\(^2\) Briefly, 120 serial 5-μm sections were cut from each artery and a set of stains performed at 50-μm steps. Stains in each set included: hematoxylin and eosin (H & E), picrosirius red to detect collagen, Verhoeff-Van Gieson to visualize medial elastin, Carstairs to detect fibrin and red blood cells, fibrinogen immunostain (Dako), and Mac-2 immunostain (Cedarlane Labs) to detect macrophages.

Images of sections were captured with a digital camera. For each stain, 6 – 8 step sections per mouse were analyzed using Image-Pro Plus software (Media Cybernetics). Internal and external elastic lamina (IEL and EEL) and lumen borders were traced. Medial area was measured
as the area between the EEL and IEL. Lesion area was measured as the area between the IEL and lumen surface. Vessel area was calculated from the EEL assuming circular geometry in vivo (area = EEL circumference² / 4π). Lumen area was calculated as: (IEL circumference² / 4π) – (lesion area). Lesion area occupied by collagen and macrophages was measured on picrosirius red and Mac-2-stained slides, using the color thresholding and planimetry functions of ImagePro. The percentage of lesions occupied by collagen or macrophages was calculated using the total lesion area measured either on a H & E-stained slide 5 μm away (for Picrosirius Red) or on the same slide (for Mac-2).

Medial elastin breaks were considered present if any of the medial elastic laminae were discontinuous. Intraplaque hemorrhage, fibrin deposition, medial elastin breaks, and fibrous cap disruption were identified by an observer blinded to genotype, using published criteria. These criteria included: fibrous cap disruption was present if at least 3 sections separated by 50 μm showed discontinuity or disruption of the fibrous cap accompanied by disruption of the underlying plaque structure. Intraplaque hemorrhage was defined as extraluminal red blood cells in at least 3 sections separated by 50 μm. Fibrin deposition was identified by the presence of both red-orange Carstairs stain and fibrin(ogen) immunostain in the same location in a minimum of 3 step sections per artery. Intraplaque hemorrhages were assessed by a second blinded observer, whose assessments were nearly identical to those of the first observer.

Sections of aortic roots were stained with H & E and Mac-2, and intimal lesion area, intimal Mac-2-stained area, and percentage Mac-2-stained area measured. The presence of intraplaque hemorrhage was evaluated using the same criteria as for the innominate analysis, only H & E-stained slides were used.

**MMP Activity Assay**

Experimental samples, including aortic-conditioned media and human neutrophil lysates (obtained from normal human volunteers according to an approved institutional protocol) were incubated with OmniMMP Fluorogenic Substrate (Biomol) at 37 °C in a black 96-well assay
plate (Corning). Substrate cleavage was measured with a fluorometer (BioTek) using an excitation filter of 328 nm and emission filter of 393 nm.

**Gelatin Zymography**
Aortic explant culture media, aortic extracts, and neutrophil lysate were separated under non-reducing conditions by 10% SDS-PAGE in gels containing 0.5 mg/ml gelatin. Neutrophil lysate was used as a positive control for MMP-9 gelatinolytic activity. Protein in explant culture media was measured (Bio-Rad) and equal amounts of protein were analyzed under nonreducing conditions. After electrophoresis at 4 °C, gels were incubated in renaturation buffer (50 mM Tris-HCl, 100 mM NaCl, 2.5% Triton X-100) for 90 min at room temperature, then in Development/Reaction Buffer (50 mM Tris-HCl, 10 mM CaCl$_2$) for 24 hrs at 37 °C. Gels were fixed and stained with Coomassie blue for 8 hrs, washed, and dried.

**Immunoprecipitation and Western Blotting**
Aortic-conditioned media and neutrophil lysate were pre-cleared with protein G Sepharose beads (Invitrogen) and incubated with an antibody to rat MMP-9 that also binds human and mouse MMP-9 (Millipore # AB19016; 1:100 dilution) or to GAPDH (Sigma) for 2 hrs on an orbital shaker. Protein G Sepharose bead slurry (20 µL) was added and incubated for 1 hr, the beads were removed by centrifugation, and supernatant was collected for MMP activity assay. The beads were washed 3 times with 800 µL ice-cold PBS, resuspended in 60 µL 2 X sample buffer, and boiled at 95 °C for 5 min. Released proteins were separated by nonreducing SDS-PAGE and blotted on a membrane. The membrane was incubated with the same MMP-9 antibody used for immunoprecipitation (1:1000 dilution), then with a secondary anti-rabbit antibody (Santa Cruz NA934VS; 1:10,000 dilution). Bound antibody was detected with ECL Western Blotting Detection reagents (GE Healthcare Life Sciences).

**Statistics**
Data are mean ± SD or median (25–75% range). A priori hypotheses were tested with the unpaired t test or by Mann-Whitney rank-sum test when group data were non-normally distributed or if group variances were unequal. Survival rate curves were generated with the Kaplan-Meier method and compared with the log-rank test. Fisher's exact test was used to compare the prevalence of intraplaque hemorrhage, fibrin deposition, and fibrous cap disruption.
**Supplemental Table**

**Table I. Innominate Artery Dimensions in Bone Marrow Recipients**

<table>
<thead>
<tr>
<th>Bone Marrow Donor Genotype:</th>
<th>SR-uPA$^{0/0}$</th>
<th>(n)</th>
<th>SR-uPA$^{+/0}$</th>
<th>(n)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessel area (mm$^2$)</td>
<td>0.42 (0.39 – 0.44)</td>
<td>(22)</td>
<td>0.42 (0.33 – 0.45)</td>
<td>(17)</td>
<td>0.8</td>
</tr>
<tr>
<td>Medial area (mm$^2$)</td>
<td>0.098 ± 0.016</td>
<td>(22)</td>
<td>0.10 ± 0.014</td>
<td>(17)</td>
<td>0.3</td>
</tr>
<tr>
<td>Lesion area (mm$^3$)</td>
<td>0.14 (0.12 – 0.16)</td>
<td>(22)</td>
<td>0.17 (0.089 – 0.19)</td>
<td>(17)</td>
<td>0.7</td>
</tr>
<tr>
<td>Lumen area (mm$^2$)</td>
<td>0.18 (0.15 – 0.19)</td>
<td>(22)</td>
<td>0.15 (0.14 – 0.17)</td>
<td>(17)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Values are mean ± SD (compared by t test) or median (25 – 75%) range (compared by Mann-Whitney test); (n) = number of recipient mice.
Figure I

Sudden death-free survival (%)

Time after BMT (days)

SR-uPA^{0/0} (28)

SR-uPA^{+/0} (37)

P = 0.001
Supplemental Figure Legends

Figure I. Survival after bone-marrow transplantation (BMT). 35-wk-old nontransgenic Apoe\(^{−/−}\) mice were lethally irradiated and transplanted with BM from either nontransgenic (SR-uPA\(^{0/0}\)) or transgenic (SR-uPA\(^{+/0}\)) Apoe\(^{−/−}\) donors. Events are sudden deaths. The number of mice entered into each group is indicated.

Figure II. Histologic and immunohistochemical analysis of serial sections of an innominate artery from a SR-uPA\(^{+/0}\) bone-marrow transplant recipient. A) Hematoxylin and eosin; B) Picrosirius red, viewed with polarized light; C) Mac-2 immunostain; D) Carstairs stain; E) Fibrin(ogen) immunostain; F) Verhoeff-Van Gieson stain. Note that in D–E, some—but not all—areas with fibrin(ogen) staining and extravascular red cells (arrows) also stain red-orange with Carstairs stain. Fibrin accumulation was judged present only when these areas were superimposable.\(^2\) Size bar = 100 μm.

Figure III. Medial elastin break in innominate artery of a bone marrow transplant recipient. A) Section showing areas of broken (B) and intact (C) elastin. B–C) Higher magnification of boxes in A. Verhoeff-Van Gieson stain. Arrow (B) = break. Size bars: A, 100 μm; B–C, 50 μm.

Figure IV. Immunoprecipitation with MMP-9 antibodies removes MMP activity from human neutrophil lysate. Lysate MMP activity was measured with a fluorogenic substrate after immunoprecipitation with antibodies to MMP-9 or GAPDH. Negative relative fluorescence unit (RFU) readings indicate fluorescence below that of background wells.
References


