Altered Vascular Injury Responses in Mice Deficient in Protease-Activated Receptor-1

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Abstract—Expression of protease-activated receptor-1 (PAR-1), a cell-surface receptor for thrombin, is increased in balloon-injured rat carotid artery and human atherosclerotic tissue. To examine the role of PAR-1 in vascular injury, we compared vascular injury responses in wild-type (WT) and PAR-1−/− mice. Arterial injury was induced by inserting a flexible guidewire into the common carotid artery and withdrawing it 6 times with rotation. Bromodeoxyuridine, delivered subcutaneously by osmotic minipump, was used to measure cellular proliferation. Mice were perfusion-fixed at 1, 2, 5, 10, and 14 days after injury. Extensive endothelial damage, mural thrombosis, platelet adherence, and medial smooth muscle cell loss and necrosis were apparent at day 1 in both WT and PAR-1−/− mice. The incidence of thrombosis or platelet deposition in WT and PAR-1−/− mice declined from 100% at day 1 to 25% and 21%, respectively, at 14 days. Endothelial disruption, as assessed by Evan’s blue uptake, was maximum at day 1 and declined by day 14. This apparent endothelial regrowth was similar in WT and PAR-1−/− mice. Significant medial thickening at 14 days after injury was similar in WT (from 22.8±1.7 to 30.7±1.9 μm) and PAR-1−/− (from 23.2±2.1 to 30.5±2.2 μm) mice. Medial area also increased in response to injury but to a lesser extent in PAR-1−/− mice (from 0.0250±0.0044 to 0.0312±0.0047 mm²) than in WT mice (from 0.0266±0.0040 to 0.0398±0.0050 mm²). Neointima was variable and occurred in 6 of 13 WT and 5 of 12 PAR-1−/− mice. However, intimal area tended to be less in PAR-1−/− mice (0.0016±0.0007 mm²) compared with WT mice (0.0082±0.0032 mm²), although this difference did not achieve statistical significance (P = 0.06). Cell density was significantly greater in normal carotids from PAR-1−/− (6.4±0.5×10³/mm²) compared with WT (4.3±0.8×10³/mm²) mice and remained elevated after injury. Vessel and lumen diameters tended to increase in WT mice after injury, whereas vessel diameter was unchanged and lumen diameter actually decreased in PAR-1−/− mice. Cell proliferation in injured carotid arteries was similar in PAR-1−/− and WT mice. These data suggest that PAR-1−/− may play a role in vascular injury responses in this mouse model via possible effects on extracellular matrix regulation. (Arterioscler Thromb Vasc Biol. 1999;19:3014-3024.)

Key Words: protease-activated receptor ■ knockout mouse ■ thrombin ■ thrombin receptor ■ cell proliferation

Several lines of evidence suggest a significant role for thrombin in vascular injury responses associated with thrombosis, atherosclerosis, and mechanical arterial injury, such as that caused by balloon angioplasty or stent implantation.1–3 Concentrations of thrombin at sites of vascular injury are significantly elevated. For example, 5 hours after balloon injury of the rabbit aorta, thrombin activity was 50 fmol · min⁻¹ · cm⁻², gradually decreasing to 10 fmol · min⁻¹ · cm⁻² at 24 hours after injury.4 These levels were sustained for at least 10 days. In human plasma, thrombin concentrations in the vicinity of a thrombus have been estimated to be as high as 140 nmol/L.5 In addition to evidence for elevated thrombin levels at sites of vascular injury, direct inhibition of thrombin by hirudin has been shown to reduce neointimal thickening induced by balloon angioplasty in normal and hypercholesterolemic rabbits and pigs and in normal rats.6–10 Surprisingly, clinical studies with the direct thrombin inhibitors hirudin11 and hirulog12 and the indirect thrombin inhibitors heparin11 and low-molecular-weight heparin13 have not demonstrated a significant effect on restenosis after angioplasty. However, these thrombin inhibitors were administered during the procedure and for only 3 to 28 days after the procedure. Long-term inhibition of thrombin may be required to produce a significant effect.

Although thrombin’s pivotal role in fibrin polymerization and clot formation contributes to the vascular injury response, activation of specific cell-surface receptors for thrombin may also participate in the vascular injury response. The thrombin receptor, or protease-activated receptor-1 (PAR-1), is a member of a novel family of G protein–coupled receptors activated by proteolytic receptor cleavage.14,15 Receptor activation results in cell signaling and a number of different cellular functions. Within the vasculature, PAR-1 activation causes alteration of vascular tone16–18; stimulation of vascular smooth muscle proliferation19; stimulation of vascular smooth muscle procollagen synthesis20; activation of endo-
thelial cells leading to release of nitric oxide,endothelin, prostaglandins, and von Willebrand factor; alteration of adhesion molecule expression; and increased vascular permeability. Several of these cellular functions, particularly stimulation of vascular smooth muscle proliferation and collagen synthesis, are integral to vascular wound healing. In addition, PAR-1 mRNA and protein expression is upregulated in response to different forms of vascular injury, such that induced by balloon angioplasty in the rat and baboon. In human advanced atherosclerotic plaques. Thus, elevated thrombin levels at sites of vascular injury can activate the upregulated levels of PAR-1, leading to a number of cellular responses that may participate in the vascular injury response.

Results of recent studies attempting to elucidate the role of PAR-1 in vascular injury responses have been conflicting. In a rabbit arterial injury model, hirudin effectively inhibited neointimal thickening in response to balloon angioplasty, but local administration of PAR-1 antisense oligodeoxynucleotides in vivo did not affect the degree of neointimal thickening in this model. In contrast, an antibody to the rat PAR-1 was shown to significantly decrease neointimal formation and PAR-1 mRNA in a rat balloon angioplasty injury model. Because PAR-1 does not mediate thrombin-induced platelet aggregation in rats and mice, the result suggests an important role for a nonplatelet PAR-1 response in vascular injury.

To further elucidate the role of PAR-1 in vascular injury, we evaluated the responses induced by mechanical injury of the carotid artery of mice made deficient in the PAR-1 gene in comparison with age- and weight-matched wild-type (WT) mice.

Methods

PAR-1–Deficient Mice

The gene for the thrombin receptor (PAR-1) was disrupted in mice by homologous recombination as described previously. As has been reported previously, these mice appear phenotypically normal on both gross and histological examinations. However, matings between PAR-1−/− mice occur less frequently and generally produce much smaller litters. The latter is apparently due to partial embryonic lethality at embryonic day 8. Genetically matched WT mice (C57BL/6) were also bred and used for these studies.

Induction of Vascular Injury

All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Care and Use Committee of The R.W. Johnson Pharmaceutical Research Institute. The left carotid artery injury was induced in 95 WT and 95 PAR-1–deficient mice by using a modification of a previously described procedure. The investigator was unaware of the genotype throughout the experiment. Statistical analysis consisted of paired t tests to compare left and right carotid arteries and unpaired t tests to compare results between genotypes. Probability values <0.05 were considered statistically significant.

Tissue Collection

At 1, 2, 5, 10, and 14 days after injury, mice were anesthetized with pentobarbital (100 mg/kg IM), and 50 μL of 5% Evan’s blue solution containing 1000 U/mL heparin was administered intravenously to demarcate the deendothelialized area of the carotid artery. Ten minutes later, the chest was opened, and a 20-gauge needle was placed in the left ventricle. Mice were perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) at a constant pressure of 100 mm Hg. The length of blue-stained carotid artery was measured. The carotid artery was cut transversely at the center of the blue-stained area. Both carotid halves were fixed in 10% neutral buffered formalin, then placed in PBS, and routinely processed for paraffin embedding. Four 5-μm-thick sections, 100 μm apart, from the central portion of each vessel half were used for morphometric analysis. Adjacent sections were processed for elastin staining or immunohistochemical labeling for BrdU and PAR-1.

van Gieson Histochemistry

For elastin staining, slides were placed in a solution containing alcoholic hematoxylin, FeCl₃, and Weigert’s iodine for 10 minutes; rinsed in distilled water; and then placed into an FeCl₃ solution for 3 minutes. Sections were rinsed in tap water and then in 95% alcohol to remove the iodine. The slides were then placed into van Gieson’s solution for 3 minutes, rinsed in 95% alcohol, dehydrated, and coverslipped.

Immunohistochemistry

After being dewaxed, the slides were microwaved in Target solution (Dako), treated with 3% H₂O₂ to block endogenous peroxidase, and incubated with 1N HCl for 30 minutes at 42°C. Slides were then washed in PBS and incubated for 60 minutes with a mouse anti-BrdU biotinylated primary antibody (anti-BrdU, Zymed) at room temperature. The slides were washed in PBS and incubated for 30 minutes with peroxidase-conjugated mouse antibody (avidin horseradish peroxidase–conjugated biotin complex, Vector Laboratories) at room temperature. After the slides were washed with PBS, diamobenzidine tetrahydrochloride (Biomedia) was added as a chromogen, and the slides were then counterstained with hematoxylin, dehydrated, and coverslipped. Negative control antibody consisted of same-species IgG isotype nonimmune serum (Vector Labs). A rabbit polyclonal antibody to PAR-1, generated by using the carboxyl terminal 10 amino acids of rat PAR-1 (The R.W. Johnson Pharmaceutical Research Institute, La Jolla, Calif), was used to assess the expression of PAR-1 in another set of sections. A biotinylated secondary antibody (goat anti-rabbit), followed by avidin-biotin–horseradish peroxidase complex reagent and 3,3′-diaminobenzidine, was used to detect the primary antibody. Slides were counterstained with hematoxylin.

Morphometry

Morphometric analyses were performed on elastin-stained tissue sections. For each animal, 8 cross sections from the injured left carotid and the uninjured right carotid arteries were viewed by using a Sony CCD video camera attached to a Leitz microscope and digitized with Image Pro image analysis software (Media Cybernetics). Representative sections were also photographed with a conventional camera for presentation in Figures. For each artery section, the area of the media and any neointima were measured. A custom program (Phase 3 Imaging Systems, Glen Mills, Pa) was used with Image Pro to measure the area and perimeter of the lumen, internal elastic lamina, and external elastic lamina. Area and average thickness of the media and neointima were derived from these measurements. BrdU-labeled and unlabeled nuclei within the media and intima were counted manually. The investigator performing the analysis was unaware of the genotype. Statistical analysis consisted of paired t tests to compare left and right carotid arteries and unpaired t tests to compare results between genotypes. Probability values <0.05 were considered statistically significant.
Results

Responses to Injury at 24 Hours

The wire-injury procedure resulted in significant endothelial denudation and damage or loss of a portion of the medial vascular smooth muscle cells at 24 hours after injury. The latter was indicated by the significant decrease in total nuclei in the injured arteries, expressed as a percent of total nuclei in the corresponding uninjured artery (P<0.001 versus uninjured artery). Acute medial cell loss was comparable in WT and PAR-1<sup>−/−</sup> mice: 69±9% and 73±4% of the uninjured carotid artery, respectively. Figures 1A and 1C show examples of uninjured carotid artery sections from a WT and a PAR-1<sup>−/−</sup> mouse, respectively, wherein the presence of a continuous, normal endothelium in both can be observed (arrows indicate endothelial nuclei). Figures 1B and 1D show examples of the vascular injury response at 24 hours, which consisted of swelling of the media, disruption and loss of smooth muscle cells, and endothelial damage. In the particular example from a PAR-1<sup>−/−</sup> mouse (Figure 1D), the degree of disruption of vascular smooth muscle cells and vacuolization were greater than in a WT mouse (Figure 1B). However, when all vessels analyzed at 24 hours were evaluated, the range of the severity of damage was similar in both genotypes. The exposed subendothelium had adherent platelet aggregates visible by light microscopy (Figures 1B and 1D, arrowheads). The degree of platelet deposition varied between animals, ranging from small platelet aggregates to extensive multilayered platelet deposition and thrombosis. However, there were no differences in these characteristics between the 2 genotypes. Fibrin and red blood cells were also sometimes observed in the media, indicating occasional medial dissection resulting from the tip of the wire.

Time Course of Thrombosis and Reendothelialization

At 24 hours after injury, all vessels had some degree of luminal thrombosis, some of which was occlusive. However,
The rate of endothelial regrowth was not different in WT and PAR-1−/− mice throughout the entire length of the vessel (aortic arch to the carotid bifurcation). Stained with Evans blue, expressed as a percentage of the vessel length of the vessel had disrupted endothelium (Figure 2, lower panel). However, from 5 to 14 days after injury, there was a gradual increase in the percentage of reendothelialization, reaching a plateau of ≈80% reendothelialization.

The incidence of thrombosis was similar in WT and PAR-1−/− arteries with visible thrombus (occlusive and nonocclusive) or microscopic evidence of platelet deposition in the lumen at different times after injury. Total number of WT mice analyzed was 8, 9, 17, 21, and 24 at 1, 2, 5, 10, and 14 days, respectively. Total number of PAR-1−/− mice analyzed was 8, 10, 14, 15, and 19 at 1, 2, 5, 10, and 14 days, respectively. Lower panel shows endothelial regrowth, as determined by the length of carotid artery not stained with Evans blue, expressed as a percentage of the entire length of the vessel (aortic arch to the carotid bifurcation). The rate of endothelial regrowth was not different in WT and PAR-1−/− mice (ANOVA).

Intravenous Evan’s blue solution administered immediately before perfusion was used to visualize the proportion of the injured vessel with disrupted (blue) and intact (white) endothelium at 1, 2, 5, 10, and 14 days after injury. Reendothelialization was expressed as the percentage of the vessel from the aortic arch to the carotid bifurcation with intact endothelium. At 1 and 2 days after injury, nearly the entire length of the vessel had disrupted endothelium (Figure 2, lower panel). However, from 5 to 14 days after injury, there was a gradual increase in the percentage of reendothelialization, reaching a plateau of ≈80% reendothelialization.

This increase presumably represents healing and regrowth of the endothelium over time. The extent or rate of endothelial regrowth was not different in PAR-1−/− mice compared with WT mice (Figure 2, lower panel).

Vascular Injury Responses at 14 Days

Figure 3 shows examples of vascular injury responses in WT mice at 14 days after vascular injury. Left-hand panels are sections stained for elastin, whereas right-hand panels are nearly adjacent sections immunolabeled with BrdU. Proliferating cells that had incorporated BrdU had brown nuclei, whereas nondividing cells had blue nuclei. Negative control antibody did not produce any detectable staining (data not shown). Figures 3A and 3B show an uninjured right carotid artery with normal medial smooth muscle layers, an intact endothelium, and the absence of BrdU-labeled nuclei (no cell proliferation). Figures 3C and 3D show a typical example of medial thickening (arrows) without significant neointima in an injured carotid artery at 14 days. Medial thickening was accompanied by a decrease in cell density but an increase in BrdU-labeled nuclei (arrowheads) in the media. The example in Figures 3E and 3F are sections from another animal in which both medial thickening and significant neointimal formation (arrowheads) were observed. Note the similar decreases in cell density in the media. BrdU-labeled nuclei were present throughout the neointima and media.

Figure 4 shows corresponding examples of the vascular injury response in PAR-1−/− deficient mice. Figures 4A and 4B show a normal, uninjured carotid artery that has a similar appearance to WT arteries. Figures 4C and 4D show an example of medial thickening with only a thin neointima (arrowheads). Figures 4E and 4F show an example from another animal in which medial thickening was accompanied by significant neointimal formation (arrowheads). In both of these examples, there was a comparable degree of medial cell loss and an increase in BrdU-labeled nuclei in the media and neointima.

Carotid arteries from both WT and PAR-1−/− mice were also subjected to morphometric analysis. The medial areas of the uninjured right carotid arteries were similar in WT and PAR-1−/− mice (Table 1, upper portion). The most consistent aspect of the response to injury at 14 days was an increase in medial thickness in both WT and PAR-1−/− arteries. The degree of increase in thickness was similar in WT and PAR-1−/− mice (Table 1, upper portion). The medial areas of injured carotid arteries were also increased in WT and PAR-1−/− mice, although the degree of increase tended to be less in PAR-1−/− mice. Thus, increased medial thickness without a comparable increase in medial area in PAR-1−/− mice would suggest an inhibition of compensatory changes in vessel dimensions. Table 1, lower portion, shows that there was a tendency for vessel and lumen diameters to increase in response to injury in WT mice. However, vessel diameter did not change and lumen diameter actually tended to decrease in response to injury in PAR-1−/− mice. Injury also induced neointima formation in both groups of mice, although this response was much less consistent and quite variable. Nonetheless, initial area was less in PAR-1−/− mice, although, this difference achieved only borderline statistical significance (P=0.06). Figure 5 summarizes the medial, intimal, and total area values for individual animals as well as the mean and SE.
Figure 3. Examples of injury responses in WT mice 14 days after vascular injury. Left-hand panels are sections labeled for elastin; right-hand panels are sections labeled for BrdU. A and B, Nearly adjacent section from the right uninjured carotid artery. C and D, Nearly adjacent sections of injured carotid artery showing primarily medial thickening without significant neointima. Note increase in medial thickness (arrows) and increase in BrdU-labeled nuclei (brown label, arrowheads). E and F, Nearly adjacent sections of injured artery from another mouse with significant neointima formation (arrowheads). Note marked neointimal formation and increased BrdU-labeled nuclei (brown-labeled nuclei) in media and neointima. Original magnification ×600.
Figure 4. Examples of injury responses in PAR-1–deficient mice 14 days after vascular injury. Left-hand panels are sections labeled for elastin; right-hand panels are sections labeled for BrdU. A and B, Nearly adjacent section from the right uninjured carotid artery. C and D, Nearly adjacent sections of injured carotid artery showing primarily medial thickening with only a thin neointima. Note increase in medial thickness (arrowheads) and increase in BrdU-labeled nuclei (brown-labeled nuclei). E and F, Nearly adjacent sections of injured artery from another mouse with significant neointima formation. Note medial thickening, marked neointimal formation (arrowheads), and increased BrdU-labeled nuclei (brown-labeled nuclei) in media and neointima. Original magnification ×600.
in the WT and PAR-1−/− groups. Note the wide range of medial and intimal areas in the WT group and the more narrow range of areas and lower mean areas in the PAR-1−/− mice.

BrdU immunostaining was used to determine the cumulative number of replicating cells in the vessel wall throughout the 14 days after injury. There were very few BrdU-labeled cells in the uninjured vessel, as would be expected. However, the cell density in normal PAR-1−/− carotid arteries was significantly greater than in WT carotid arteries (Table 2). The vascular injury procedure caused significant loss of total nuclei in the media of the injured arteries compared with the corresponding uninjured artery. This phenomenon was reflected by the significant decrease in cell density after injury in both genotypes. The degree of decreased cell density was comparable in both groups, although cell density remained significantly higher in PAR-1−/− compared with WT mice. Vessel and lumen diameters tended to increase in WT mice in response to injury; vessel diameter did not change, and lumen diameter decreased in PAR-1−/− mice. P values for differences between genotypes are indicated in parentheses.

### PAR-1−/− Labeling in Injured Carotid Artery

Figure 6 shows an example of immunohistochemical labeling of PAR-1 in uninjured and injured mouse carotid arteries from a WT and a PAR-1−/− mouse. In WT mice, the PAR-1 label was not apparent in the uninjured carotid artery (Figure 6B) but was present throughout the neointima of the injured carotid artery, particularly at its luminal edge (Figure 6A). Label was absent in both the uninjured and injured carotid arteries from a PAR-1−/− mouse (Figures 6C and 6D). This indicates external elastic lamina. Values are mean±SE for n=13 WT and n=12 PAR-1−/− mice. There were no significant differences in uninjured medial thickness or area in WT and PAR-1−/− mice. Injury-induced medial thickening was significant (P<0.05) and similar in WT and PAR-1−/− groups, but medial, intimal, and total areas of the injured carotid arteries tended to be lower in PAR-1−/− compared with WT mice. Vessel and lumen diameters tended to increase in WT mice in response to injury; vessel diameter did not change, and lumen diameter decreased in PAR-1−/− mice. P values for differences between genotypes are indicated in parentheses.

<table>
<thead>
<tr>
<th>Vessel wall dimensions</th>
<th>n Uninjured Media</th>
<th>Injured Media</th>
<th>Neointima</th>
<th>Total</th>
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<tr>
<td>Thickness, μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT</td>
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<td>30.7±1.9</td>
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<td>PAR-1−/−</td>
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<tr>
<td>Area, mm²</td>
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<tr>
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<td>0.0398±0.0050</td>
<td>0.0082±0.0032</td>
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<tr>
<td>PAR-1−/−</td>
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<td>0.0250±0.0044</td>
<td>0.0312±0.0047</td>
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<table>
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<tr>
<th>EEL Diameter, μm</th>
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<th>Injured</th>
</tr>
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<tbody>
<tr>
<td>Vessel and lumen diameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
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</tr>
<tr>
<td>PAR-1−/−</td>
<td>12</td>
<td>348±30</td>
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Table 1. Morphometric Analysis of Carotid Arteries 14 Days After Vascular Injury in Wild-Type (WT) and PAR-1−/− Mice

Figure 5. Morphometric analysis of arterial injury responses in WT and PAR-1−/− mice 14 days after vascular injury. Morphometric analysis was performed on 4 sections per artery spaced 100 μm apart. Areas were then averaged. Medial area, intimal area, and total areas from each animal (WT, filled circles; PAR-1−/−, filled triangles) are plotted along with the mean±SE for each group (WT, open circle; PAR-1−/−, open triangle). Medial, intimal, and total areas tended to be less in PAR-1−/− mice, but this effect was not statistically significant.
Table 2. Cell Density and Proliferative Response 14 Days After Vascular Injury in Wild-Type (WT) and PAR-1–Deficient (PAR-1−/−) Mice

<table>
<thead>
<tr>
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<th>Injured Media</th>
<th>Neointima</th>
<th>Total Vessel Wall</th>
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<tr>
<td></td>
<td>Cell Density, % Labeled</td>
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<td></td>
<td>×10^3/mm^2</td>
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<td>×10^3/mm^2</td>
<td>×10^3/mm^2</td>
</tr>
<tr>
<td>WT</td>
<td>4.3±0.8</td>
<td>2.3±0.4*</td>
<td>10.3±7.8*</td>
<td>2.6±0.3*</td>
</tr>
<tr>
<td>PAR-1−/−</td>
<td>6.4±0.5†</td>
<td>3.1±0.5*</td>
<td>22.2±3.2*</td>
<td>4.0±0.5†</td>
</tr>
</tbody>
</table>

Values are mean±SE for n=8 WT and n=12 PAR-1−/− mice. Cell density is the average number of nuclei per mm² based on the analysis of 4 sections per animal. Cell density in uninjured carotid arteries from PAR-1−/− mice was significantly greater than that in WT mice. Cell density was significantly decreased in injured carotid arteries in both WT and PAR-1−/− mice. Total cell density remained elevated in injured carotid arteries from PAR-1−/− mice compared with WT mice.

*P<0.05, significant change compared with uninjured.
†P<0.05, significantly different from WT.

Discussion

Using mice made deficient in PAR-1, a novel cellular receptor activated by thrombin,14 we investigated the role of PAR-1 in the response to vascular injury induced by mechanical damage to the lumen of the carotid artery. Our results suggest that the absence of PAR-1 alters the vascular injury response in this mouse model. Neointimal thickening was blunted in PAR-1–deficient mice. Although medial thickening in response to injury was comparable in WT and PAR-1–deficient mice, the corresponding increase in total medial area in WT mice tended to be less in PAR-1–deficient mice. In addition, although vessel and lumen diameters tended to increase after injury, vessel diameter was unchanged and lumen diameter actually decreased in PAR-1–deficient mice. Moreover, the reduction in lumen diameter in PAR-1–deficient mice occurred despite the fact that neointimal thickening was less. This profile of vessel wall changes is consistent with a role for PAR-1 in positive remodeling after vascular injury.

The vascular injury response comprises several cellular processes, including thrombosis, platelet adhesion, cell migration, cell proliferation, and matrix remodeling. We evaluated and quantified several components of the injury response in our model. Thrombosis, either occlusive or nonocclusive, and histological evidence of platelet deposition were prominent within 24 hours of injury, as is found after vascular injury in other animal models37,38 and in humans.39 The incidence of thrombosis and platelet deposition was not different in WT and PAR-1–deficient mice. This finding was not unexpected, since thrombin-induced platelet activation in mice and coagulation in general do not involve PAR-1.34,35 Early occlusive and nonocclusive thrombosis resolved over time. The rate of resolution was not different in WT and PAR-1–deficient mice. The occlusion rate at 14 days was ~20% and did not differ in WT and PAR-1–deficient mice. Therefore, the influence of thrombosis and associated mediators, such as thrombin and platelet-derived growth factor, was equivalent in the 2 genotypes and thus, does not explain the observed differences in vascular injury response. The rate and extent of endothelial regrowth and the return of barrier function after injury are other critical determinants of the vascular injury response. Rapid endothelial regrowth has been shown to limit neointimal formation.40 Endothelial regrowth, which presumably reflects both cell migration and proliferation, was unaffected by the absence of PAR-1, indicating that differences in the rate of endothelial regrowth do not account for the differences in vascular injury response. Proliferation plays a critical role in the vascular injury response and in neointimal thickening. However, the proportion of proliferating cells in the media, as assessed by BrdU labeling over the entire 14 days after injury, was not different in WT and PAR-1–deficient mice. All cells in the neointima of WT and PAR-1–deficient mice were BrdU labeled, as would be expected given the continuous 14-day BrdU infusion that was used. Thus, true proliferation rates in the intima could not be determined with this BrdU labeling protocol. However, based on proliferation rates in the media, the absence of PAR-1 does not appear to affect vascular smooth muscle proliferation, suggesting that the effects of PAR-1 deficiency on vascular injury responses are not a result of altered proliferation.

PAR-1 deficiency may also affect tissue remodeling, including cell growth and matrix degradation and synthesis. Normal vessels from PAR-1–deficient mice had a significantly greater smooth muscle cell density compared with that in WT mice. Even though vascular injury decreased cell density in the media of both genotypes, cell density in the PAR-1–deficient mice remained greater than that in WT mice. Cell density in the neointima was also greater in PAR-1–deficient mice. The increased cell density in normal vessels may have resulted from reduced matrix synthesis and secretion during development and/or reduced cell growth rate and volume. Thrombin, acting via PAR-1, has been shown to stimulate collagen synthesis in vascular smooth muscle cells,20 activate matrix metalloproteinases, and increase their synthesis in endothelial cells.41 Thus, thrombin and PAR-1 appear to be involved in matrix remodeling. The greater cell density associated with reduced neointimal area in PAR-1–deficient mice suggests compromised extracellular matrix formation during the injury response. In addition, positive remodeling associated with vascular injury must also involve matrix breakdown, synthesis, and secretion. The tendency for inhibition of positive remodeling in PAR-1–deficient mice after injury may also reflect a deficiency in matrix synthesis and secretion, matrix degradation, and/or alteration in the control of cell growth.

A great deal of evidence implicates thrombin as well as PAR-1 as pivotal mediators of the vascular injury response.1–3 Besides the identified functional roles of thrombin...
and PAR-1 in platelet aggregation, cell proliferation, and matrix synthesis, both thrombin and PAR-1 expression and message appear to be upregulated after vascular injury in animal models\(^27\) and in human atherosclerotic vessels.\(^28,29\)

We have shown that PAR-1 is also upregulated in the mouse carotid artery in response to vascular injury. Inhibition of thrombin’s enzymatic function has been shown to reduce vascular injury response in various animal models.\(^6–10\) However, thrombin’s actions involve both pure enzymatic functions as well as activation of its cellular receptor, PAR-1. Therefore, results of studies with thrombin inhibitors do not necessarily prove a role for PAR-1 in vascular injury. The lack of small-molecule PAR-1 antagonists has made it difficult to define a specific role for PAR-1 in vascular injury. Nonetheless, other approaches have been employed to interfere with PAR-1 activation. In a recent study, antisense oligodeoxynucleotide to PAR-1 was used to assess the role of PAR-1 in a rabbit model of vascular injury.\(^30\) Local administration of this antisense oligodeoxynucleotide appeared to inhibit expression of PAR-1 mRNA in vivo. However, there was no effect on the degree of intimal thickening. In the same model, hirudin, a direct inhibitor of thrombin, significantly reduced the intimal thickening response.\(^30\) Thus, the authors concluded that although thrombin may be involved in the injury response, it did not appear that PAR-1 was involved in this animal model. However, in another study in a rat model, an antibody specific for PAR-1 was shown to inhibit the intimal thickening response at antibody concentrations that effectively inhibited thrombin-induced vascular smooth muscle proliferation in vitro.\(^31\) Hirudin was also effective in this model. These results support a role for thrombin and PAR-1 in the vascular injury response in a rat model.

Differences in the apparent participation of PAR-1 in vascular injury responses may clearly result from species and
model differences. For example, although PAR-1 mediates thrombin-induced platelet aggregation in humans, PAR-1 is not expressed in mouse, rat, and rabbit platelets and thus, does not mediate thrombin-induced platelet aggregation in these species. The response to thrombin in mouse and rat platelets appears to be mediated by PAR-3 and PAR-4.2,44-46,2 distinct protease-activated thrombin receptors. Because platelet adhesion, aggregation, and degranulation are important components of the vascular injury process, these platelet-dependent processes would not be affected in PAR-1–deficient mice. Therefore, our mouse model does not assess the contribution of thrombin-induced platelet aggregation to the vascular injury response. Model-dependent differences in injury responses are likely. Whereas balloon-injury techniques used in rats and rabbits induce a uniform stretch and circumferential denudation, the wire injury does not induce uniform stretch and causes a more focal luminal damage. The resultant injury response may be quite different from the response to balloon angioplasty in rabbits and rats or the process of restenosis in patients with atherosclerosis undergoing balloon angioplasty. Despite these differences, the mouse model does involve several fundamental components of vascular wound repair that are common to this type of injury response in humans. Unfortunately, the focal nature of the lesion, coupled with the relatively thin mouse carotid artery, resulted in great variability, particularly in the incidence of intimal thickening. This variability limited the sensitivity of the model to detect changes in the injury response. Nonetheless, several trends for differences in various aspects of the injury response in PAR-1–deficient mice, taken together, suggest a role for PAR-1 in vascular injury.

Findings in transgenically manipulated mice, in which the gene for a single protein has been deleted, must be interpreted with caution, since cellular and/or physiological changes compensating for the absent protein may occur. Partial embryonic survival in PAR-1–deficient mice may involve such a compensation. In addition, the accentuated cellular response to platelet-deployed growth factor in fibroblasts from PAR-1–deficient mice also represents a secondary compensation that may mask the absence of PAR-1. The increased vascular smooth muscle cell density in PAR-1–deficient mice, which may be a consequence of the lack of PAR-1 during development, will tend to alter the vascular injury response. Thus, the apparent inhibition of positive remodeling in PAR-1–deficient mice may be a consequence of the higher baseline cell density rather than the absence of PAR-1. Finally, the response to vascular injury involves other important factors besides PAR-1. Many of these factors and pathways are multiply redundant and could compensate for PAR-1’s absence.

In summary, our results suggest that in this mouse model, PAR-1 may play a role in regulation of vascular matrix formation and remodeling associated with vascular injury. Determination of the ultimate significance of PAR-1 in the vascular injury response will require further study, including identification and application of small-molecule PAR-1 antagonists both in animal models and eventually in humans.

References


Altered Vascular Injury Responses in Mice Deficient in Protease-Activated Receptor-1
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