

Plasminogen Activator Inhibitor-1 From Bone Marrow-Derived Cells Suppresses Neointimal Formation After Vascular Injury in Mice

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Objective—To investigate the ability of bone marrow (BM)-derived cells to modulate neointimal growth after injury by expressing plasminogen activator inhibitor-1 (PAI-1).

Methods and Results—We performed BM transplantation (BMT) in lethally irradiated wild-type (WT) and PAI-1^{-/-} mice. Three weeks after carotid injury with ferric chloride, analysis of Y-chromosome DNA expression in the vessel wall of female hosts revealed that 20.8±6.0% of the cells in the neointima and 37.6±5.7% of those in the media were of BM origin. Lack of PAI-1 in either the host or the donor cells did not affect recruitment of BM-derived cells into sites of vascular injury. The neointima consisted predominantly of smooth muscle cells, and a proportion of these cells expressed PAI-1. Overall, lack of PAI-1 was associated with enhanced neointimal formation. However, importantly, BMT^{WT→PAI-1^{-/-}} mice exhibited reduced neointimal area ($P=0.05$) and luminal stenosis ($P=0.04$) compared with BMT^{PAI-1^{-/-}→PAI-1^{-/-}} mice. Although PAI-1-expressing cells were shown to be present in BMT^{WT→PAI-1^{-/-}} lesions, these mice did not exhibit detectable levels of the inhibitor in the circulation, suggesting that local production of PAI-1 by cells in the neointima and media was sufficient to reduce luminal stenosis.

Conclusions—PAI-1 from BM-derived cells appears capable of suppressing neointimal growth after vascular injury. (*Arterioscler Thromb Vasc Biol.* 2006;26:1254-1259.)

Key Words: bone marrow-derived vascular progenitor cells ■ mouse model ■ plasminogen activator inhibitor ■ vascular injury

Recent evidence suggests that the vascular wound healing response to injury may involve not only the migration of local smooth muscle cells (SMCs) from neighboring “healthy” tissue but also the recruitment of circulating vascular progenitor cells from the bone marrow (BM) into the vessel wall.¹⁻³ The mechanisms by which BM-derived SMCs modulate neointimal growth remain largely unknown, but we hypothesized that plasminogen activator inhibitor-1 (PAI-1) could be one of the mediators of this process. In fact, PAI-1 is expressed by vascular endothelial and SMCs,⁴ and its expression is upregulated in human atherosclerotic lesions⁵ and during the vascular wound healing response to experimental injury.⁶ Previous studies that attempted to clarify the role of PAI-1 in neointimal formation using various models of arterial injury in gene-inactivated mice yielded rather contradicting findings,⁷⁻¹¹ but the apparent discrepancies need to be interpreted in the context of the pleiotropic effects of the inhibitor on vascular cells and the surrounding matrix.¹² For example, PAI-1 stabilizes arterial thrombi in vivo⁶

and may thus have provided a provisional matrix facilitating cell migration, at least in injury models characterized by heavy deposition of fibrin.⁸ However, under different experimental conditions,^{7,11} PAI-1 expression in the vessel wall may have contributed to reduced cell motility by inhibiting both pericellular plasmin-mediated proteolysis and the attachment of migrating cells to the extracellular matrix via integrins and the urokinase receptor.¹²

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In the present study, we investigated the possible role of PAI-1 in mediating at least some of the effects of BM-derived cells on vascular remodeling after injury. Whole body irradiation and BM transplantation (BMT) experiments were performed in wild-type (WT) and PAI-1-deficient mice (Jackson Laboratories, Bar Harbor, Me), followed by induction of arterial injury using the ferric chloride injury model.^{6,13,14} Apart from quantitatively assessing and characterizing the BM-derived cells involved in the remodeling process,

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we examined their ability to express PAI-1 and sought to determine whether the expression of the inhibitor by cells of BM origin might help modulate (ie, contain neointimal growth and luminal stenosis in this experimental model of severe damage to the vessel wall).

Methods

The Methods are provided as supplemental online material, available at <http://atvb.ahajournals.org>.

Results

Irradiation and BMT Does Not Alter the Effects of PAI-1 on Arterial Thrombosis and Neointimal Growth

WT or PAI-1-deficient (PAI-1^{-/-}) mice were lethally irradiated and transplanted with unfractionated WT or PAI-1^{-/-} BM (10⁶ cells). Four weeks after BMT (ie, after allowing for complete BM reconstitution), carotid artery injury was induced using ferric chloride.^{4,6} The following mouse groups were studied: WT mice transplanted with WT BM (BMT^{WT→WT}; n=8); WT mice that received PAI-1^{-/-} BM (BMT^{PAI-1^{-/-}→WT}; n=14); PAI-1^{-/-} mice with PAI-1^{-/-} BM (BMT^{PAI-1^{-/-}→PAI-1^{-/-}}; n=12); and PAI-1^{-/-} with WT BM (BMT^{WT→PAI-1^{-/-}}; n=12).

We have previously shown that lack of PAI-1 in mice is associated with formation of unstable thrombi and prolongation of the time to thrombotic occlusion after injury.^{4,6} In the present study, BMT^{PAI-1^{-/-}→PAI-1^{-/-}} also tended to have prolonged times to complete occlusion compared with BMT^{WT→WT} mice (15.3±1.5 versus 13.2±1.8 minutes), although the difference did not reach statistical significance. The patency rates 25 minutes after injury were 38% in BMT^{PAI-1^{-/-}→PAI-1^{-/-}} compared with 12% in BMT^{WT→WT} mice (*P*=0.3). Moreover, the mean time to thrombotic occlusion was almost identical between mice of the BMT^{WT→WT} group and a control group of 15 nonirradiated, nontransplanted WT mice (13.2±1.8 versus 13.7±0.9 minutes; *P*=0.95), supporting the conclusion that irradiation or BMT per se did not appear to affect the thrombotic response of WT and PAI-1^{-/-} mice to arterial injury.

Three weeks after injury, carotid vessels were harvested and the extent of neointimal formation was quantitatively assessed.⁴ Lack of PAI-1 was associated with enhanced neointimal formation after injury (representative findings shown in Figure I, available online at <http://atvb.ahajournals.org>). Overall, the neointimal area was larger in BMT^{PAI-1^{-/-}→PAI-1^{-/-}} compared with BMT^{WT→WT} mice (5525±852 versus 2907±493 μm²; *P*=0.04), and luminal stenosis increased from 5.7±1.0% in BMT^{WT→WT} to 13.6±2.3% in BMT^{PAI-1^{-/-}→PAI-1^{-/-}} mice (*P*=0.02; results summarized in Figure II, available online at <http://atvb.ahajournals.org>). Of note, these differences were similar to those observed between nonirradiated, nontransplanted WT (n=9) and PAI-1^{-/-} (n=13) controls. In these latter studies, neointimal area was 4865±1173 μm² in WT mice compared with 8084±1096 μm² in their PAI-1^{-/-} counterparts (*P*=0.06), and luminal stenosis 7.0±1.0% versus 14.9±2.7% (*P*=0.03). Thus, PAI-1 suppressed neointimal growth after ferric chloride-induced injury. Our data further suggest that whole body irradiation slightly (but

not significantly) reduced neointimal growth and luminal stenosis both in WT and in PAI-1^{-/-} mice.

Presence of BM-Derived Cells in the Vessel Wall

Recent evidence suggests that BM-derived vascular progenitor cells participate to a varying extent in the wound healing response after mechanical injury of mouse arteries.^{1,15} To assess the presence of these cells in the neointima in the ferric chloride injury model, 2 different approaches were used. First, female animals were irradiated and transplanted with unfractionated BM harvested from the tibia and femur of male mice. After BM reconstitution and 3 weeks after injury, BM-derived (male) cells in the vessel wall were detected using in situ hybridization for Y-chromosome DNA. Second, BM from β-galactosidase transgenic (ROSA26) mice was transplanted into irradiated LacZ-WT mice. BM-derived cells in the vessel wall were identified using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) as a substrate for detection of β-galactosidase enzyme activity. As shown in Figure 1, BM-derived cells could be found in the neointima (Figure 1a and 1b) and media (Figure 1c and 1d) but also in the adventitia, where they were shown to line perivascular small blood vessels (Figure 1e and 1f). Quantitative analysis of Y-chromosome-positive and LacZ-positive cells showed that the 2 methods yielded comparable results and a similar proportion of BM-derived cells in the vessel wall (Table). Of note, neither method detected donor (Y-DNA- or LacZ-positive) cells in uninjured carotid arteries (Figure 1g).

Spatial and Temporal Distribution of BM-Derived Cells

Analysis of serial cross-sections through the injured segment (data not shown) and longitudinal sections (Figure 1h) revealed that the presence of BM-derived cells was most pronounced at the center of the injury and gradually decreased toward its proximal and distal borders. To assess the time course of migration of BM-derived cells into the neointima, additional experiments were performed using BM from β-galactosidase-transgenic mice. LacZ-positive cells could not be detected in the vessel wall 30 minutes, 24 hours, or 1 week after injury, despite the abundance of BM-derived (LacZ-positive) cells in the vascular lumen (Figure 2a through 2d). In fact, the absence of BM-derived cells coincided with the complete loss of SMCs in the media for up to 1 week after the severe injury induced to the vessel wall by ferric chloride. Thus, migration of circulating BM-derived cells into the vessel wall occurred relatively late during the remodeling process, between the first and the third week after injury.

Characterization of Vascular Cells of BM Origin

In accordance with previous observations,⁶ immunostaining for von Willebrand factor (vWF) and α-actin confirmed that 3 weeks after injury, the endothelial cell layer had been completely reconstituted, and the neointima consisted predominantly of vascular SMCs. Double staining revealed that vWF+/LacZ+ (endothelial) cells were only occasionally detected (Figure 3a and 3b), whereas a substantial proportion of the LacZ-positive cells in the

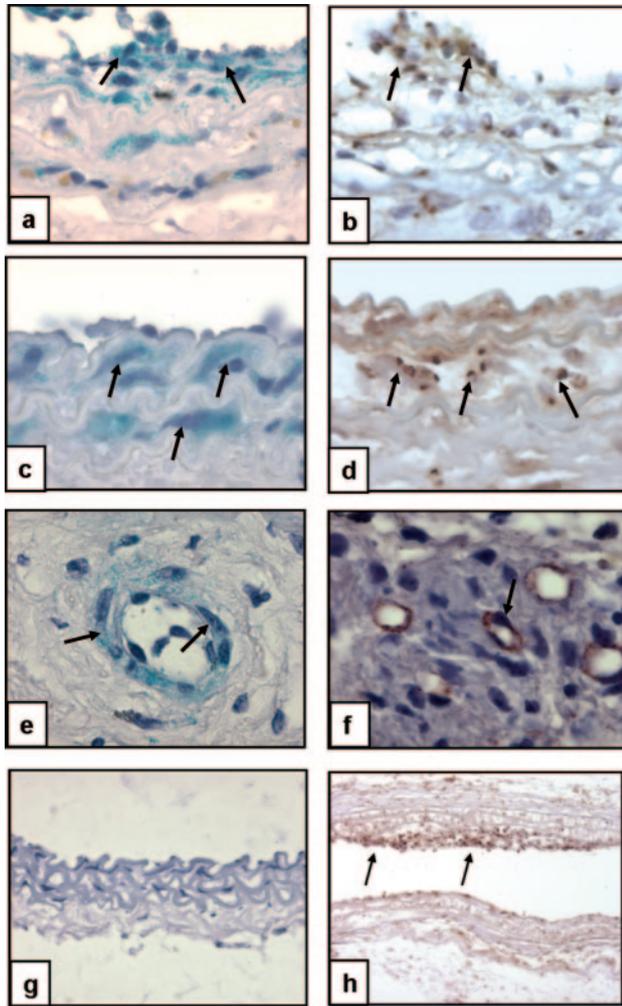


Figure 1. Detection of BM-derived cells in the vessel wall 3 weeks after injury. Displayed are representative arterial sections demonstrating the presence of β -galactosidase-positive (arrows indicate examples of turquoise blue cytoplasm in a, c, and e), or Y-chromosome-positive (arrows indicate eccentric brown nuclear stain in b, d, f, and h) cells in the neointima (top row), media (second row), and adventitia (third row). g shows absence of β -galactosidase staining in an uninjured artery of a WT mouse transplanted with LacZ^{+/+} BM 3 weeks after injury to the contralateral carotid artery. Finally, in h, accumulation of Y-chromosome-positive cells is shown on a longitudinal section through the injured segment. Magnification $\times 1000$ in a through f; $\times 400$ in g and h.

neointima and media also stained positive for α -actin ($30.6 \pm 5.0\%$; range 5% to 60%; Figure 3c and 3d) and, to a smaller extent, smooth muscle-myosin heavy chain (Figure 3e). In contrast, Mac3-positive tissue macrophages could be clearly distinguished from LacZ-positive cells within the media (Figure 3f).

BM-Derived Cells Express PAI-1 in Vascular Lesions

Immunohistochemical analysis of PAI-1 antigen expression showed that, as opposed to BMT^{PAI-1^{-/-}→PAI-1^{-/-}} mice, both BMT^{WT→WT} and BMT^{PAI-1^{-/-}→WT} mice expressed PAI-1 in the vessel wall 3 weeks after injury (data not shown). The PAI-1-immunopositive area was reduced from $20.5 \pm 1.0\%$ in BMT^{WT→WT} to $7.3 \pm 2.4\%$ in BMT^{PAI-1^{-/-}→WT} mice ($P < 0.001$). However, importantly, transplantation of WT BM into PAI-1^{-/-} mice (BMT^{WT→PAI-1^{-/-}}) resulted in the presence of PAI-1-expressing cells in the neointima (immunopositive area $6.3 \pm 1.1\%$). PAI-1-positive cells already appeared in the vessel wall 1 week after injury (Figure 2e and 2f), and at 3 weeks, PAI-1 immunoreactivity in lesions correlated significantly with the LacZ-positive area ($r = 0.78$; $P = 0.022$). Using immunofluorescence double labeling of frozen sections, the cells expressing PAI-1 could be identified as CD31- or vWF-positive endothelial cells (Figure 4a and 4b) and α -actin-positive (data not shown) or myosin heavy chain-positive SMCs (Figure 4c). Of note, it is unlikely that the PAI-1 detected in the vessel wall had been released from circulating platelets during or after injury because we showed in a previous⁴ and in the present (Figure III, available online at <http://atvb.ahajournals.org>) study that, in contrast to human platelets, mouse platelets do not contain detectable amounts of PAI-1. Moreover, measurement of circulating PAI-1 antigen revealed a mean concentration of 3.5 ± 1.1 ng/mL in plasma from BMT^{WT→WT} mice, whereas PAI-1 antigen was undetectable not only in BMT^{PAI-1^{-/-}→PAI-1^{-/-}} but also in 9 of 10 BMT^{WT→PAI-1^{-/-}} mice (mean concentration 0.11 ± 0.11 ng/mL).

Finally, our studies revealed that lack of PAI-1 in either the host or the donor did not significantly affect the proportion of BM-derived cells in vascular lesions, as demonstrated by quantitative comparison of Y chromosome-positive donor cells in female recipients of the 4 mouse groups (BMT^{WT→WT}, BMT^{PAI-1^{-/-}→WT}, BMT^{PAI-1^{-/-}→PAI-1^{-/-}}, and BMT^{WT→PAI-1^{-/-}}; data not shown).

PAI-1 Expressed By BM-Derived Cells May Suppress Neointimal Formation

We investigated the functional role of BM-derived cells in the neointima and their possible contribution to vascular remodeling in relation to PAI-1 expression. As mentioned above and shown in Figure I, lack of PAI-1 was associated with enhanced neointimal formation after ferric chloride-induced injury. Figure I further shows that neither the neointimal area nor the severity of luminal stenosis was significantly affected in WT mice receiving PAI-1^{-/-} BM compared with BMT^{WT→WT} mice ($P = 0.70$ and 0.89 , respectively; Figure II). On the other hand,

BM-Derived Cells in the Vessel Wall 3 Weeks After Ferric Chloride-Induced Injury

	LacZ-Positive Cells		Y-DNA-Positive Cells	
	No.	Percentage of Total Cells	No.	Percentage of Total Cells
Neointima	5.2 ± 2.8	$21.6 \pm 6.9\%$ (range 3% to 48%)	2.6 ± 0.5	$20.8 \pm 6.0\%$ (range 4% to 64%)
Media	18.4 ± 7.4	$36.9 \pm 13.0\%$ (range 0.1% to 82%)	19.4 ± 2.9	$37.6 \pm 5.7\%$ (range 13% to 61%)

The numbers of LacZ-positive or Y-DNA-positive cells in the vessel wall were determined as explained in the Methods. Numbers shown represent mean values \pm SEM.

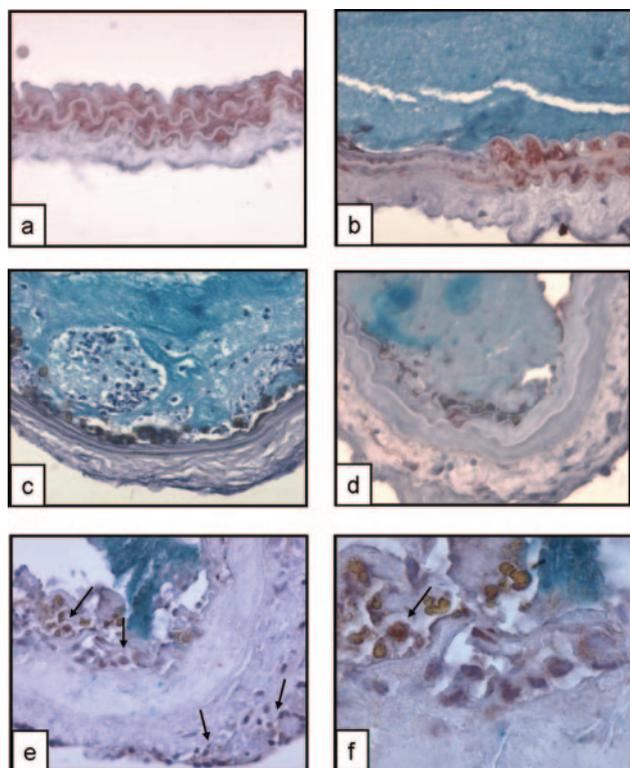


Figure 2. Time course of migration of LacZ-positive cells into the vessel wall. WT mice were transplanted with LacZ^{+/+} BM, and arterial injury was induced as described in the Methods. Carotid arteries were harvested, and β -galactosidase activity (to identify donor, ie, BM-derived cells) as well as α -actin (to localize SMCs; a through d) and PAI-1 (e and f) antigen were detected histochemically. Displayed are representative sections of an uninjured (contralateral) artery (a), and at different times after injury (b, 30 minutes; c, 24 hours; d through f, 1 week). Magnification $\times 400$ (a through e) and $\times 1000$ (f). Arrows point to PAI-1-positive cells in the adventitia and in the organizing thrombus.

transplantation of PAI-1-expressing (ie, WT BM cells into PAI-1^{-/-}) mice resulted in marked reduction both of neointimal size (3416 ± 595 versus $5525 \pm 852 \mu\text{m}^2$; $P=0.05$) and the degree of luminal stenosis (7.7 ± 1.4 versus $13.6 \pm 2.3\%$; $P=0.04$) compared with BMT^{PAI-1^{-/-}→PAI-1^{-/-}} mice (Figure I; summarized in Figure II). Thus, PAI-1 expressed by BM-derived cells appeared to be capable of suppressing, at least in part, neointimal growth after arterial injury.

Discussion

Proliferation and migration of vascular SMCs plays a crucial role in intimal hyperplasia and is a common feature of atherosclerotic lesions. The origin of neointimal SMCs is diverse;¹⁶ besides endothelial transdifferentiation^{17,18} and the migration of dedifferentiated medial SMCs or adventitial myofibroblasts into the neointima,¹⁹ it may involve the recruitment of vascular progenitor cells from the BM and the circulation.^{20,21} In fact, BM-derived progenitor cells have been detected both in human^{22,23} and in murine^{1,24} vascular lesions.

In the present study, we examined whether, and to what extent, BM-derived cells participate in the wound healing response to vascular injury. The spatial and temporal pattern

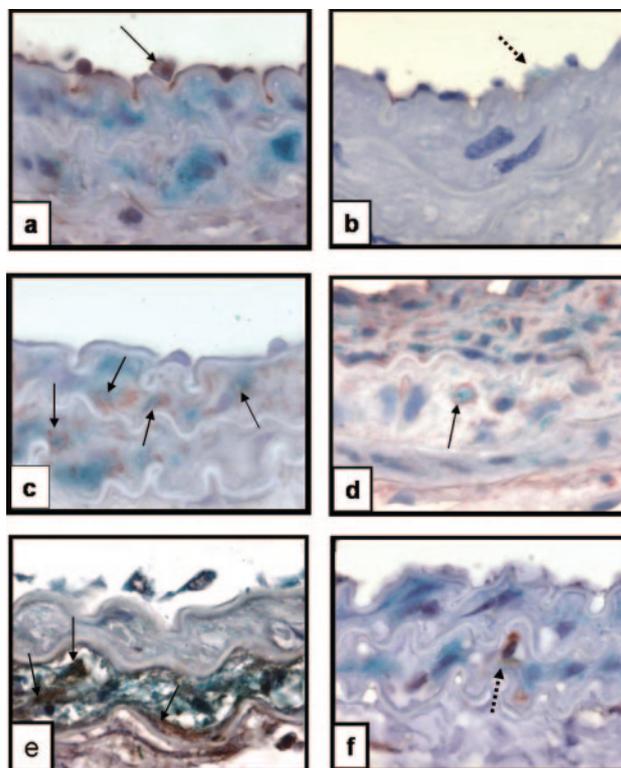


Figure 3. Immunohistochemical characterization of BM-derived cells in the vessel wall 3 weeks after injury. Endothelial cells were detected using antibodies against vWF (a and b), vascular SMCs using antibodies against α -actin (c and d) and myosin heavy chain (e), and tissue macrophages using antibodies against Mac-3 (f). Arrows with a continuous line point to LacZ-positive, vWF-immunopositive (a), α -actin-immunopositive (c and d), or smooth muscle myosin heavy chain-immunopositive cells. Arrows with a dashed line highlight LacZ-positive, vWF-immunonegative (b), or Mac-3-immunonegative (f) cells. Magnification $\times 1000$.

of the recruitment of BM-derived cells into the vessel wall was studied, and the functional consequences of this process were investigated, focusing on the role of PAI-1. Based on the demonstration of β -galactosidase activity in WT mice, or the detection of male (Y-chromosome DNA-positive) donor cells in female mice, we identified $\approx 21\%$ of the cells in the neointima and 37% of the cells in the media as being of BM origin 3 weeks after injury. In our study, Y-DNA in situ hybridization and X-gal staining both yielded similar results, but it has been suggested that the number of Y-DNA-positive cells may be underestimated by in situ hybridization because of the small and eccentric nuclear staining pattern.²⁵ Importantly, several (31%) of the LacZ-positive BM-derived cells also stained positive for α -actin, suggesting that they had transdifferentiated into vascular SMCs 3 weeks after injury.

Both the mean proportion of BM-derived cells in the vessel wall and the wide range of values observed in individual mice after ferric chloride injury are in accordance with previous studies that applied various models of mechanically induced arterial injury using LacZ^{1,3} or green fluorescent protein transgenic¹⁵ mice or gender-mismatched BMT.^{1,26} Together, our results and those of other authors suggest that the type and severity of vascular injury is an important determinant of the extent of progenitor cell recruitment.¹⁵ Thus, the relatively

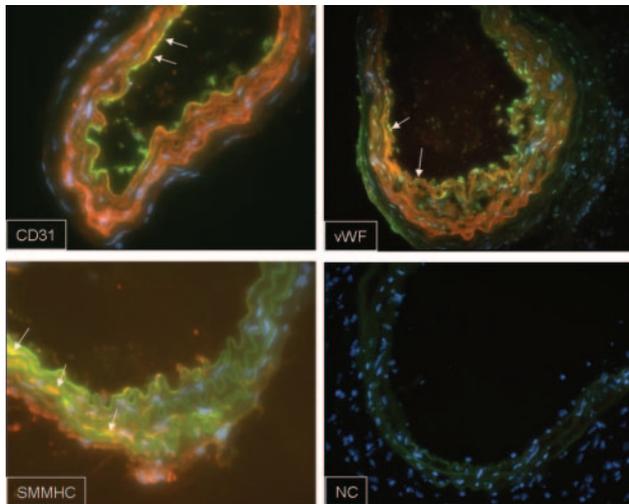


Figure 4. Detection of PAI-1-expressing cells in the neointima of BMT $WT \rightarrow PAI-1^{-/-}$ mice. Frozen cross-sections through the neointima of BMT $WT \rightarrow PAI-1^{-/-}$ mice ($n=3$) were analyzed for the expression of PAI-1 antigen (red) as well as the presence of CD31 (green) and vWF (green) as endothelial cell marker, or smooth muscle myosin heavy chain (SMMHC; green). Arrows point to double positive cells (yellow). Bottom right panel shows the negative control (NC). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). Magnification $\times 400$.

high percentage of Y-DNA- or LacZ-positive cells in our studies is consistent with the observation that ferric chloride induces extensive endothelial denudation and medial cell loss over ≥ 1 week.⁶ Of course, experimental injury models, including the one used in the present study, cannot exactly reproduce the pathophysiology of human atherosclerosis, atherothrombosis, or restenosis. Nevertheless, injury with ferric chloride is characterized by the reproducible formation of platelet-rich arterial thrombi, and the lesions that develop in mice during the remodeling process exhibit histological similarities with human atherosclerotic plaques.^{6,13}

Because our studies were performed in mice that had been lethally irradiated and then reconstituted with unfractionated whole BM, the exact phenotype of the progenitor cells that gave rise to vascular SMCs cannot not be determined. Previous studies suggested that endothelial and mural cells are derived from a common progenitor,²⁷ whereas others suggested the presence of separate progenitors for endothelial and SMCs.²⁸ Vascular progenitor cells appear to reside within the hematopoietic stem cell fraction, and hematopoietic stem cells were shown to differentiate into SMCs after seeding on primary rat aortic SMCs.¹ Notably, BM stromal cells also have the potential to differentiate into SMCs.^{29,30} On the other hand, the non-BM-derived (LacZ-negative or Y-DNA-negative) cells found in neointimal lesions in our study could have originated from dedifferentiation, migration, and proliferation of resident cells in the media or adventitia.¹⁹ However, it cannot be excluded that radio-resistant progenitor cells within the vessel wall may also have been involved in the remodeling process.²⁴

Although the present study and previous reports strongly support the recruitment of BM-derived cells into vascular lesions, the functional contribution of these cells to the remodeling process remains controversial to date. To begin to

dissect the mechanisms mediating the effects of BM-derived cells in the wall of injured vessels, we examined the potential of BM-derived cells to produce PAI-1 and thus possibly modulate extracellular matrix proteolysis, cell adhesion, and migration. In accordance with a previous model of severe injury to the vessel wall,⁷ lack of PAI-1 was associated with significantly enhanced neointimal growth and luminal stenosis after ferric chloride injury, and this effect was not altered by irradiation or BMT. Thus, the modulation of neointimal growth by the inhibitor appeared to result predominantly from its direct (inhibitory) effects on cell migration.¹² However, a number of studies, some of which yielded contradicting results despite using very similar injury models,^{7,10,11,31} suggest that the inhibitory effects of PAI-1 on neointimal growth may be neutralized, or even reversed, by its thrombus-stabilizing effects, depending on the severity and the extent of the thrombotic response, which may be highly variable in vivo.³² Notably, the effects of PAI-1 on neointimal growth do not appear to involve modulation of the recruitment of BM-derived cells into sites of vascular injury because we found that lack of PAI-1 in either the host or the donor cells did not affect the proportion of BM-derived cells in the vessel wall.

In support of a role of BM-derived cells in modulating neointimal growth, we found that the increase of neointimal formation in mice lacking PAI-1 was almost completely prevented in the presence of PAI-1-expressing BM-derived cells in the vessel wall. Based on our findings in LacZ chimeras and gender-mismatched mice, and on the detection of PAI-1-immunopositive cells in the vessel wall of PAI-1-deficient host mice, the production of PAI-1 by less than one third of the cells in the neointima and the media appeared sufficient to restore the WT phenotype (ie, suppress neointimal growth and reduce luminal stenosis). It is very unlikely that PAI-1 released from circulating (donor) platelets contributed to these effects because it was shown previously^{4,33} and in the present study (supplemental data) that PAI-1 protein is undetectable in mouse platelets as opposed to the relatively high PAI-1 levels in human platelets.³⁴ Moreover, our finding that transplantation of WT (ie, PAI-1-expressing) BM cells did not result in detectable levels of the inhibitor in the circulation of PAI-1 $^{-/-}$ mice adds further support to the importance of local (vascular) versus systemic PAI-1 for vascular remodeling.

In conclusion, the present study extends previous findings by showing that BM-derived cells are recruited into, and form a substantial component of, vascular lesions developing in mice after injury. Moreover, our results suggest that these cells are capable of regulating cell migration and the vascular remodeling process, and that these effects may be related, at least in part, to the expression of protease inhibitors such as PAI-1. This experimental approach may prove useful for dissecting the mechanisms mediating the (presumed) pleiotropic effects of BM-derived progenitor cells on vascular homeostasis. In addition, the modification of the expression profile of recruited progenitor cells may represent a novel strategy for preventing the restenotic process after arterial injury.

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Arteriosclerosis, Thrombosis, and Vascular Biology



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Plasminogen Activator Inhibitor-1 From Bone Marrow–Derived Cells Suppresses Neointimal Formation After Vascular Injury in Mice

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Methods

Experimental Animals

Male and female C57Bl/6 (stock #000664), PAI-1 deficient (stock #002507) and Gt(Rosa)26Sor (stock #002192) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained at the Animal Facility of the University of Goettingen, Germany. All animal care and experimental procedures were approved by the Animal Research Committee of the University of Goettingen and complied with national guidelines for the care and use of laboratory animals.

Irradiation and Bone Marrow Transplantation

For bone marrow transplantation experiments, mice received a single dose of 9 Gy whole body irradiation from a linear accelerator (VARIAN, Palo Alto, CA, USA) at the Department of Radiotherapy and Radiooncology at the University of Goettingen, Germany. Eighteen hours later, mice were transplanted with unfractionated whole bone marrow (10^6 freshly harvested cells in a volume of 150 μ L sterile PBS) via tail vein injection. Mice were housed in isolation containers with filtered air for the following 4 weeks and inspected daily. In the absence of sufficient bone marrow engraftment, mice invariably died within the first 7 to 14 days after irradiation. In a subset of mice, successful engraftment was confirmed by demonstrating β -galactosidase activity in bone marrow and peripheral blood cells.

Carotid Injury in Mice

Mice were anesthetized with isoflurane (Forene®, Abbott, Wiesbaden, Germany) and subjected to carotid artery injury using 10% ferric chloride (FeCl_3) according to an established protocol.^{16,17} Carotid blood flow was monitored before and for 25 minutes after injury. Three weeks after injury, tissue harvest and processing was performed as previously described.¹⁸ Briefly, anesthetized mice were carefully perfusion-fixed with 4% zinc formaline through the

left ventricle. The carotid artery including the bifurcation was excised, post-fixed in 4% zinc formalin, dehydrated and embedded in paraffin wax for subsequent histochemical analysis. If β -galactosidase enzyme activity had to be preserved, animals were perfused with normal saline and the freshly excised tissue immediately processed as described below.

Analysis of PAI-1 Antigen Levels in Plasma

At the time of tissue harvest, whole blood was obtained by cardiac puncture and plasma PAI-1 antigen levels were determined by ELISA following the manufacturer's instructions (Molecular Innovations, Southfield, MI, USA).

Histochemical Studies

To visualize β -galactosidase enzyme activity in LacZ-WT mice transplanted with BM from LacZ-transgenic mice, arteries were incubated in a staining solution containing 1 mg/mL 4-Cl-5-Br-3-indolyl- β -galactosidase (X-Gal; Sigma, Taufkirchen, Germany), 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.01% sodiumdeoxycholate and 0.02% NP-40 in PBS at 37°C overnight, followed by formalin fixation and paraffin embedding. Enzyme activity was detected on 5 μ m-thick paraffin sections counterstained with hematoxyline. The number of LacZ-positive cells within the neointima or media was counted manually by two investigators and expressed as percentage of total cells. A total of 7 animals were evaluated in these experiments and the results averaged. The values obtained in each mouse represented the average of measurements performed on 4-7 arterial sections.

In Situ Hybridization

Y-chromosome positive cells within the neointima of female WT or PAI-1^{-/-} mice transplanted with BM from male WT or PAI-1^{-/-} mice were detected using the STARFISH whole chromosome paint (Cambio, Cambridge, UK). Briefly, 5- μ m thick paraffin sections

were dewaxed in a series of graded alcohols, rehydrated for 5 min in PBS and incubated in 1 M sodium thiocyanate for 10 min at 80°C. Sections were digested in 0.4% pepsin/0.1 M HCl for 10 min at 37°C and quenched by incubation for 2 min in 0.2% glycine in 2X PBS. Then, sections were post-fixed for 2 min at 4°C in 4% paraformaldehyde, washed 3 x 5 min in PBS and rehydrated to 98% ethanol. A biotinylated Y-chromosome paint (dilution, 1:15 in DNA hybridization puffer; Dako, Hamburg, Germany) was added to the sections, sealed under a coverslip using rubber cement and heated for 10 min at 60°C. Hybridization was performed overnight at 37°C in a humid chamber. On the next day, sections were washed 3 x 10 min in 50% formamide/50% 2X SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 3 x 10 min in 2X SSC and 10 min in 4X SSC/0.05% Tween-20 (all at 37°C) followed by incubation for 30 min with peroxidase link (ABC kit, Vector Laboratories, Burlingame, CA, USA) at room temperature. Slides were developed using NovaRed (Vector), counterstained with Mayer's hematoxyline, cleared through 95% and 100% ethanol and xylene substituent (HistoClear, Shandon, Dreieich, Germany) and mounted with VectaMount (Vector). The number of Y-DNA-positive cells within the neointima or media was counted manually by two investigators and expressed as percentage of total cells. As with the histochemical studies, a total of 4-7 animals per group were evaluated in these experiments. The values obtained in each mouse represented the average of measurements performed on 4-7 arterial sections. Of note, evaluation of normal male mice revealed that the sensitivity for the detection of Y-chromosome-positive cells is less than 100%, and therefore the real number of Y-chromosome-positive cells could be about 1.3-fold higher than detected. No Y-chromosome-positive cells were detected in female mice or if the DNA probe was omitted.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded and frozen carotid artery sections. Endothelial cells were visualized using a rabbit anti-mouse von Willebrand factor

(vWF) antibody (Dako, dilution 1:100 for 1 hr at 37°C) or a rat anti-mouse CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution, 1:50). Tissue macrophages were detected using a rat anti-mouse Mac-3 antibody (BD PharMingen, Heidelberg, Germany; dilution, 1:200). Plasminogen activator inhibitor-1 was localized using a polyclonal rabbit anti-mouse PAI-1 antibodies (SantaCruz; dilution, 1:20). The PAI-1 immunopositive area was evaluated in 4-5 mice per group using ImagePro Plus software (Media Cybernetics) and expressed as percentage of the total vessel wall area. The presence of smooth muscle cells (SMC) was assessed using monoclonal anti-mouse α -actin (Dako; dilution, 1:50) or anti-mouse SM myosin heavy chain antibodies (Dako; dilution 1:20 after pretreatment with 0.05% Triton-X in PBS). The number of α -actin-/ LacZ double-positive cells was evaluated on 3 mice, 5 sections per mouse, and expressed as percentage of the total number of LacZ-positive cells.

Morphometric Evaluation of Neointimal Formation

For morphometric analysis, paraffin sections were stained with Verhoeff's elastic stain and analyzed using image analysis software (ImagePro Plus). Five consecutive sections equally spaced through the injured segment were evaluated and the results averaged for each animal.

Statistical Analysis

For comparison of the times to thrombotic occlusion after injury, the Mann-Whitney non-parametric test was used. Mouse vessels that did not occlude completely were regarded as having times to thrombosis corresponding to the end of the flow monitoring period (25 minutes). For all other continuous variables, differences between mean values were tested by the two-sided Student's t test for unpaired means. Statistical significance was assumed when *P* reached a value less than 0.05.

Figure I. Effect of PAI-1 and bone marrow transplantation on the vascular wound

healing response. Vascular injury was induced in wild-type (WT) or PAI-1^{-/-} mice 4 weeks after irradiation and bone marrow (BM) transplantation. Injured arteries were harvested 3 weeks later and Verhoeff's elastica-stained paraffin sections were analyzed. The figure panels show representative findings obtained in the four mouse groups as indicated. Magnification, x400.

Figure 1

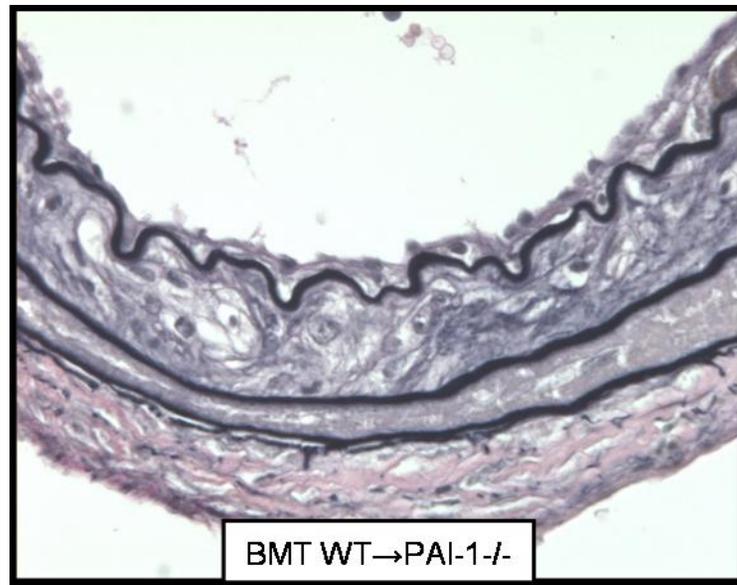
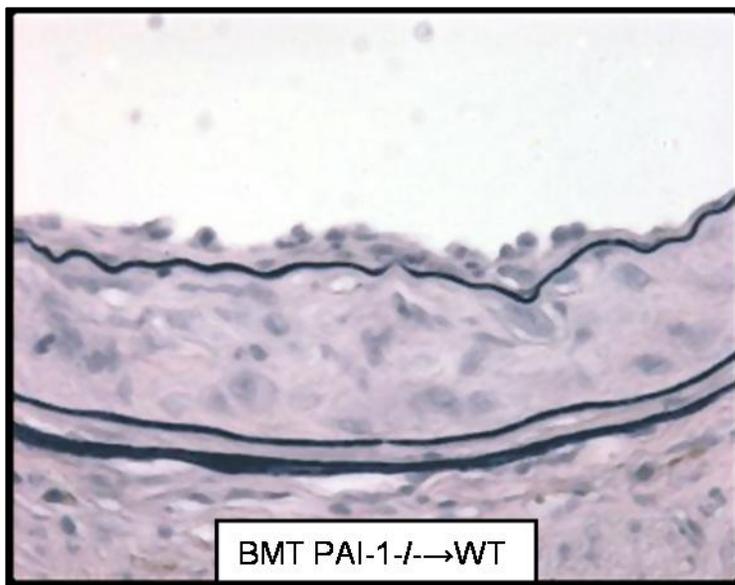
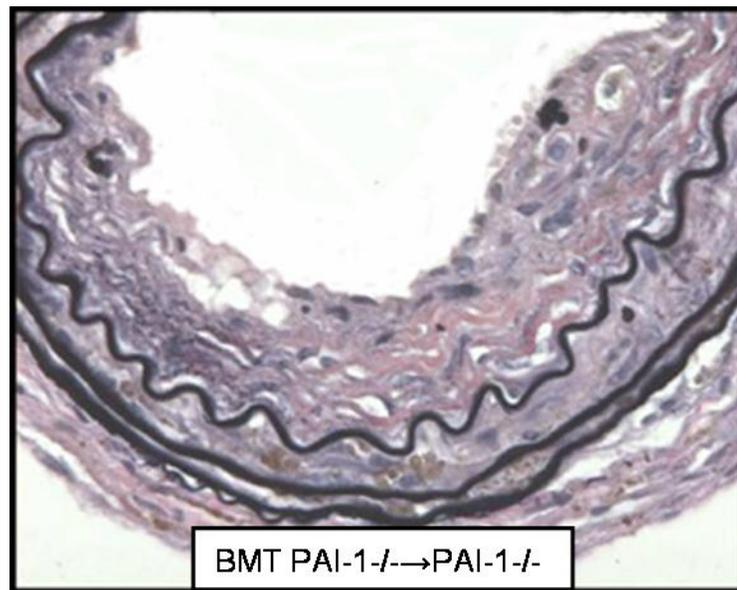
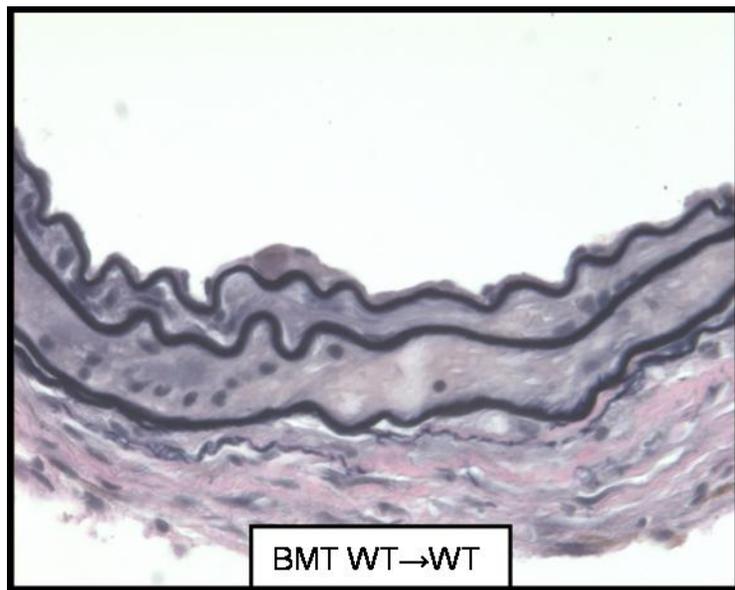


Figure II. Morphometric quantification of neointimal formation. The neointima area (panel A) and the percentage of luminal stenosis (panel B) were determined in WT mice with WT BM (n=7), WT with PAI-1^{-/-} BM (n=10), PAI-1^{-/-} with PAI-1^{-/-} BM (n=11), and PAI-1^{-/-} with WT BM (n=11) 3 weeks after induction of vascular injury. Individual values for each mouse are shown; the horizontal line through each grouping represents the mean value. The results of the Student's t-test are indicated in the graphs.

Figure II

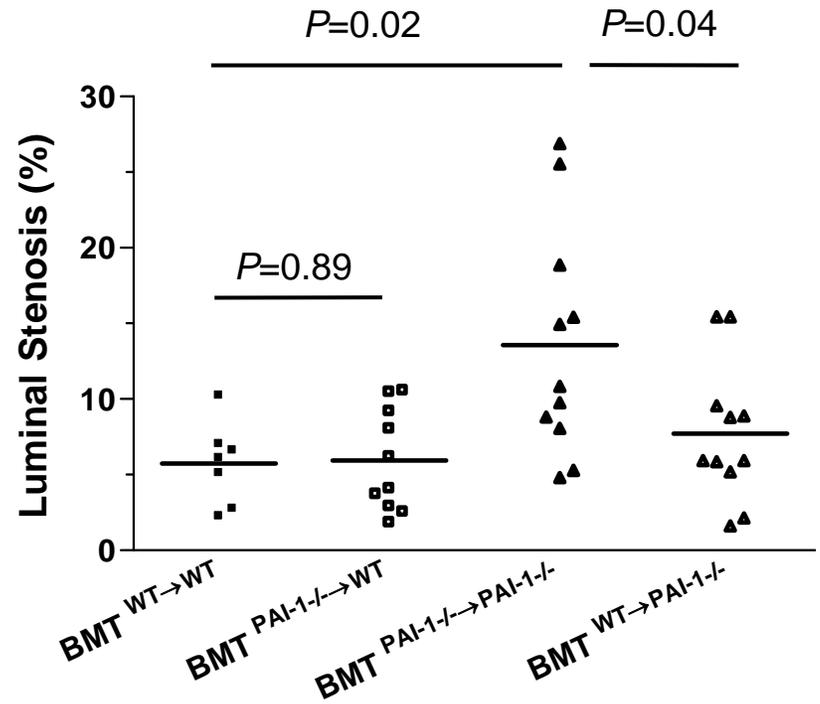
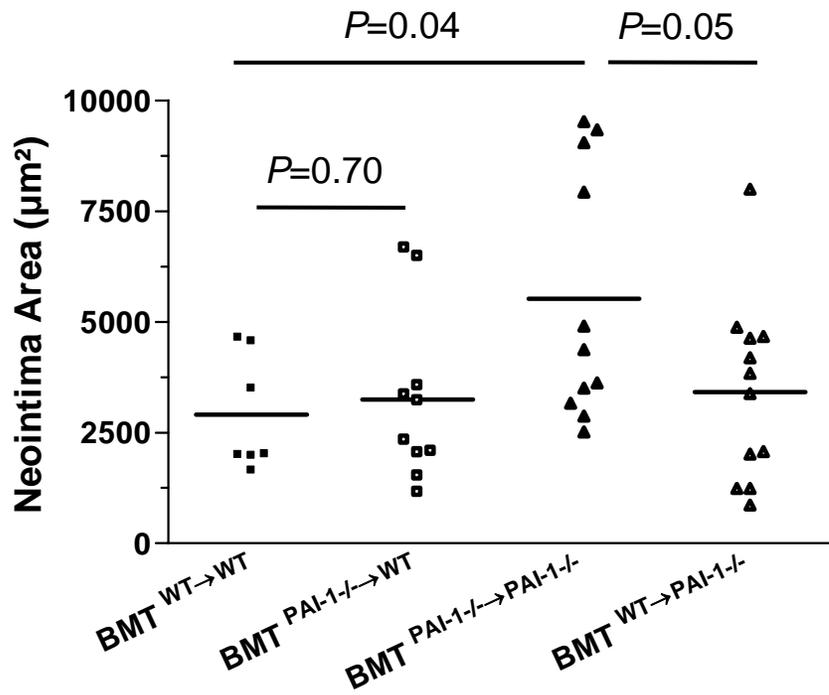


Figure III. Western blot for detection of plasminogen activator inhibitor-1 (PAI-1) in human (Hu), wild-type mouse (WT), and PAI-1-knockout mouse (PAI-1^{-/-}) platelets. Each lane was loaded with an equal amount (50 µg) of protein. PAI-1 was detected with a polyclonal rabbit anti-human/mouse PAI-1 antibody (H-135; Santa Cruz, dilution 1:1,000). PAI-1 was readily detected in human, but not in WT mouse platelets.

Figure III

Hu WT PAI-1^{-/-}



Figure IV

