The Role of Protease-Activated Receptor-1 in Bone Healing

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Protease-activated receptor (PAR)-1, a G-protein-coupled receptor activated by thrombin, mediates thrombin-induced proliferation of osteoblasts. The current study was undertaken to define the role of PAR-1 in bone repair. Holes were drilled transversely through the diaphysis of both tibiae of PAR-1-null and wild-type mice. Three days later, fewer cells had invaded the drill site from adjacent bone marrow in PAR-1-null mice than in wild-type mice, and a lower percentage of cells were labeled with [³H]thymidine in PAR-1-null drill sites. More osteoclasts were also observed in the drill site of PAR-1-null mice than in wild-type mice 7 days after drilling. New mineralized bone area was less in the drill site and on the adjacent periosteal surface in PAR-1-null mice than in wild-type mice at day 9. From day 14, no obvious differences could be seen between PAR-1-null and wild-type tibiae. *In vitro* thrombin caused a dose-dependent increase in proliferation of bone marrow stromal cells isolated from wild-type mice but not PAR-1-null mice. Thrombin stimulated survival of bone marrow stromal cells from both wild-type and PAR-1-null mice, but it did not affect bone marrow stromal cell migration in either wild-type or PAR-1-null cells. The results indicate that PAR-1 plays an early role in bone repair. (Am J Pathol 2005, 166:857–868)

Thrombin is a serine protease, which plays a central role in blood coagulation through its cleavage of fibrinogen, but also exerts specific receptor-mediated effects on cell function. Three thrombin receptors have been identified, protease-activated receptors (PARs)-1, -3, and -4, which are members of the seven transmembrane domain G-protein-coupled receptor family.^{1,2} Protease-activated

receptor-1 is expressed by osteoblasts (bone-forming cells), and mediates thrombin-induced proliferation of these cells.^{3–5} Thrombin also stimulates osteoclastic bone resorption *in vitro*.^{6,7} Thrombin is generated during tissue injury, and PAR-1 activation appears to be involved in pathological processes including inflammation and wound healing.^{8–10} For example, when thrombin or a PAR-1-activating peptide is applied to incisional skin wounds in rats, wound healing is accelerated.¹¹ Because both osteoblast proliferation and bone resorption are important components of the process of bone repair, we hypothesized that thrombin, through PAR-1, may participate in this process. The current study was undertaken to investigate this hypothesis, using mice with a targeted disruption of the PAR-1 gene.¹² Fifty percent of these mice die at about day 9.5 of gestation, and the remainder survive to become apparently normal adults. Embryonic death of PAR-1-null mice can be prevented by targeted expression of PAR-1 in endothelial cells.¹³ Although PAR-1 is expressed by platelets and mediates platelet aggregation in humans, PAR-1 is not expressed by mouse platelets, and therefore is not required for normal blood coagulation in this species.¹⁴

A number of models of bone repair have been established for use in mice, including an unstabilized fracture model, stabilized fracture models involving internal or external fixation, and models involving bone defects without fracture.^{15–21} Similar responses to bone injury are seen in all of these models, with an initial phase of cellular invasion into the defect, followed by formation of callus tissue composed of woven bone with varying amounts of cartilage. The callus is then modeled over time through the actions of osteoclasts and osteoblasts, to restore lamellar bone and normal architecture.

In the current study, we have used PAR-1-null and the corresponding wild-type (WT) mice in a model of bone repair recently established in our laboratory.²¹ In this model, a hole is drilled transversely through the full di-

Supported by the National Health and Medical Research Council of Australia (Project Grant 251575 and Program Grant 284233).

Accepted for publication November 9, 2004.

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ameter of the tibia. The drill site fills rapidly with a hematoma, and over the ensuing days the hematoma is invaded by cells from the adjacent bone marrow. These cells produce trabecular woven bone which fills the drill site by day 7. The woven bone is modeled from about day 9, to restore normal bone architecture within about four weeks.²¹ We observed a number of differences between the drill sites of PAR-1-null and WT mice during the early stages of repair. With the aim of elucidating the functional basis for some of these differences, *in vitro* studies of bone marrow cells isolated from PAR-1-null and WT mice have also been conducted.

Materials and Methods

Materials

Purified human α -thrombin was prepared as described.²² Cell culture media and additives were obtained from Invitrogen (Melbourne, Australia). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Mice in which the protease activated-receptor-1 gene had been disrupted by homologous recombination¹² (PAR-1-null mice) were kindly provided by Dr. S. R. Coughlin (University of California, San Francisco). These mice have been extensively back-crossed on the C57BL/6J background. Age-matched C57BL/6J mice were used as WT controls. All work involving animals was approved by the Animal Experimentation Ethics Committee of the School of Veterinary Science, University of Melbourne.

Surgery

Surgery was performed on 12- to 13-week-old PAR-1-null and wild-type male mice as described.²¹ Briefly, a single hole, 0.5 mm in diameter, was drilled with a 25-gauge needle. The defect passed through the entire diameter of the tibia from the medial to the lateral aspect just proximal to the distal end of the tibial crest. Surgery was carried out on both hind limbs. At various times postdrilling (0, 1, 2, 3, 5, 7, 9, 14, 28, and 42 days), mice were weighed, killed by cervical dislocation and the tibia and fibula along with the associated muscle were excised.

Histology

The left tibia was fixed for 1.5 hours in 4% paraformaldehyde in PBS and processed for preparation of cryosections. The right tibia was fixed in 70% ethanol and processed for embedding in LR-White-Hard (London Resin Company, Reading, UK). Some tissues were then demineralized in 0.33 mol/L ethylenediaminetetraacetic acid disodium salt (pH 7.4), whereas others were processed undemineralized. Transverse cryosections (10 μ m thick) were prepared and stained for the presence of tartrate-resistant acid phosphatase (TRAP) to identify osteoclasts, as described.²¹ Tissues were embedded in LR-White according to the manufacturer's instructions.

Transverse sections (5 μ m) were cut using a slab microtome with a tungsten knife (Polycut E, Leica, Bannockburn, IL). Double-sided adhesive tape was used to attach undemineralized sections to glass slides. Demineralized sections were spread using 40% acetone and 1% benzoyl alcohol, collected on 3-aminopropyltriethoxysilane-coated glass slides, then stained with Epoxy Stain (ProSciTech, Thuringowa Central, QLD, Australia). Undemineralized sections were stained with Von Kossa.

Histomorphometric Analysis

Histomorphometric analyses were conducted on images of sections captured with a digital camera (Spot, Diagnostic Instrument Inc, Sterling Heights MI) linked to an Olympus BX 60 microscope. All cell counts and area and length measurements were made with Image-Pro Plus (Media Cybernetics, Silver Spring, MD) image analysis software.

The tibial cortical bone width and total cortical bone area were measured on Epoxy-stained demineralized plastic sections of undrilled tibiae of 12-week old mice. The sections used for these measurements were at the same level of the tibia as used for drilling in the bone repair model. The sections of fibula present in the same sections were used for measurement of osteoblast surface and eroded surface as a percentage of total endosteal and periosteal surface. In TRAP-stained cryosections from 12-week-old mice, osteoclasts on endosteal and periosteal surfaces of the fibula were counted manually as TRAP-positive cells with more than two nuclei; osteoclast counts were expressed as cells/mm bone surface. Three to four sections from each animal (4 WT and 4 PAR-1-null mice) were used for histomorphometric analyses of uninjured bone.

For cell counts and new bone area measurement in sections of drilled tibiae, images were captured at different regions within the drill site and on the periosteal surface, as defined in Figure 1. Two to three sections from each animal (5 WT and 5 PAR-1-null mice for each time point) were used for cell counts and bone measurement. Demineralized epoxy-stained plastic sections through the drill site were used. Total cell number in each field at different regions within the drill site (defined in Figure 1) was counted manually using a 40x objective. Osteoclasts were counted manually as TRAP-positive cells, with more than two nuclei, adherent to bone surfaces, in demineralized TRAP-stained transverse cryosections through the drill site.

For autoradiographic studies *in vivo*, three 12- to 13-week-old PAR-1-null and WT male mice were drilled; [³H]thymidine (1 μ Ci/g; Amersham, Buckinghamshire, UK) was injected intraperitoneally 42 hours after drilling and then mice were killed at day 3 (30 hours after [³H]thymidine injection). Both tibiae were collected and used for production of demineralized plastic sections as described above. After being dipped in Ilford nuclear research emulsion K.5 (Knutsford, Cheshire, UK), sections were stored in the dark for 5 weeks at 4°C, developed in Kodak GBX (Coburg, Victoria, Australia), then stained

Figure 1. Demonstration of the regions used for cell counts and new bone area measurements. Masson's trichrome-stained plastic demineralized transverse section through the site of injury in a mouse tibia nine days after drilling. Rectangles show the different regions used: lateral side (A), bone marrow region (B), and medial side (C) of the drill site, and the adjacent periosteal surface (D). Arrows indicate the edges of the drill site; arrowheads indicate the outline of the periosteal new bone. Scale bar = 400 μ m.

with 1:100 epoxy stain. Three or four sections from each animal were used for cell counts. Total cell number and labeled nuclei (nuclei containing more than 10 grains) were counted. Results for labeled cells were expressed as the percentage of total cells in each region.

For bone area measurement, undemineralized Von Kossa-stained plastic sections through the drill sites were used. Total new trabecular bone area (Von Kossa-positive) was measured in each field. Results for new trabecular bone area are expressed as a percentage of total tissue area.

Bone Marrow Stromal Cell Isolation

Single cell suspensions of bone marrow stromal cells (BMSCs) were prepared from left and right femurs and tibiae of 4–5-week-old mice, as described.²³ Cells were pelleted, resuspended, and counted using the trypan blue exclusion method in a hemocytometer to determine the total number of viable marrow cells obtained from both femurs and tibiae. Cells were seeded into 75 cm² flasks for growth. To separate adherent stromal cells from nonadherent hemopoietic cells, medium was changed after 48 hours, removing all nonadherent cells together with the culture medium; adherent stromal cells were washed twice with PBS. Cells were fed every two or three days until confluent, then trypsinized and plated in appropriate vessels for further experiments. Cells were cultured in minimal α -essential medium containing 10% fetal calf serum, gentamicin (50 μ g/ml), amphotericin B (2.5 μ g/ml), and sodium ascorbate (50 μ g/ml) at 37°C under 5% CO₂ in air.

Colony-Forming Unit-Fibroblast Assay

Freshly isolated single cell suspensions of bone marrow were plated in 60-mm dishes at a density of 10⁵ cells/cm². To maintain the influence of nonadherent hemopoietic cells, only one third of the volume of medium was removed and replaced on days 3 and 6. On day 9, the dishes were washed with PBS, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes, and stained for alkaline phosphatase (ALP) activity using an ALP staining kit (Sigma) following the manufacturer's instructions. Colonies of cells containing more than 20 cells were counted manually as colony-forming units-fibroblast, to be referred to as CFU-Fs. The number of ALP-positive CFU-Fs ($\geq 20\%$ ALP-positive cells) was also counted.

Apoptosis Assay

Apoptotic cells were identified on the basis of nuclear morphology with 4,6-diamidino-2 phenylindole (DAPI) staining. Cells were plated in eight-chamber slides, grown to confluence and then deprived of serum for 24 hours before treatment with thrombin for 72 hours. Cell monolayers were fixed, permeabilized and mounted in Gelvatol (25% polyvinyl alcohol, 30% glycerol, and 0.1% sodium azide) containing DAPI (1 μ g/ml). Fluorescent images were captured and cell counts conducted with Image Pro Plus software; total and apoptotic nuclei were counted in two or three representative fields per well using a 20 $\times$ objective. Total cells were counted automatically, and apoptotic cells in the same field were counted by eye, identified on the basis of morphology (nuclear condensation and fragmentation). Values for apoptotic cells were expressed as the percentage ($\pm$ SEM; $n = 4$) of total cells.

Cell Proliferation Assay

Cell proliferation was assayed by 5-bromo-2'-deoxyuridine (BrdU) incorporation using a kit (Roche, Nutley, NJ USA). Proliferation assays for WT and PAR-1-null BMSCs were always carried out at the same time, and initial plating cell densities were the same in WT and PAR-1-null mice. BMSCs were cultured in 96-well plates until approximately 80% confluent. Cells were washed and deprived of serum for 24 hours and then treated with thrombin in serum-free medium for an additional 24 hours; BrdU labeling solution was added simultaneously with thrombin. Cells were fixed, and incorporated BrdU was detected by a direct immunoperoxidase method and tetramethylbenzidine substrate, according to the instructions supplied with the kit. The absorbance was read at 450 nm and 690 nm (in dual wavelength mode) in a Labsystems (Helsinki, Finland) Multiskan MS plate reader.

Migration Assay

The migration assays were performed in 24-well Transwell cell culture plates (Corning Inc., Corning, NY) with

polycarbonate filters (6.5 mm in diameter with 8 μ m pores). Red blood cells were removed from freshly isolated bone marrow cells using 0.84% NH_4Cl , then cells were washed with PBS and suspended at $2 \times 10^6/\text{ml}$ in serum-free medium. The lower chambers of Transwell plates were filled with 0.6 ml of serum-free medium with or without 100 nmol/L thrombin, and the upper chambers with 0.1 ml of cell suspension. The cells were allowed to migrate for 5 hours at 37°C in a humidified atmosphere with 5% CO_2 in air. The inserts were carefully removed. Nonadherent cells within the lower chambers were collected with their medium, and viable cells were counted using the trypan blue exclusion method in a hemocytometer. Adherent cells were fixed with 4% paraformaldehyde in PBS and stained with Mayer's hematoxylin. Adherent cells in five representative fields/well were counted under the microscope.

RNA Isolation

RNA was isolated from first passage BMSCs, long bone and bone marrow using the following method. RNA was isolated from the diaphyseal region of tibiae and femurs which had been stripped of all attendant soft tissue, and from which the metaphyses had been removed and bone marrow expelled with PBS. The diaphyseal regions of tibiae and femurs were washed thoroughly with PBS flushed from a 21-gauge needle. Bones were snap frozen in liquid nitrogen and ground to a fine powder before the addition of Tri-Reagent; first passage BMSCs and bone marrow were lysed directly in Tri-Reagent. RNA was extracted from the samples using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Polymerase chain reaction was used to investigate the expression of thrombin receptors and prothrombin. The first strand cDNA synthesis was carried out on total RNA using M-MLV reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI). Primers for the mouse PAR and glyceraldehyde-3-phosphate (GAPDH) genes have been described elsewhere.²⁴ The sequence for the prothrombin primers was as follows: forward, CTA TGT CGC ACG TCC GC; reverse, GCA CTG GAT ACC GGT ATG G (product size 392 bp). The PCR reactions were carried out using the following thermal profile: 95°C for 30 seconds, annealing for 30 seconds at the appropriate temperature (55°C for PAR-1 and PAR-4; 51°C for PAR-3 and prothrombin) extension at 72°C for 30 seconds, for 30 cycles followed by a final extension step for 5 minutes at 72°C. Mouse liver cDNA was used as a positive control to confirm that the primers could detect expression of mouse prothrombin.

Real-time PCR was carried out to investigate the relative expression of PAR-3 and PAR-4 in bone marrow isolated from WT and PAR-1 null mice (3 mice of each genotype). The sequence for the real-time PAR-3 and

Table 1. Histomorphometry of Cortical Bone in the Fibula of PAR-1-Null and WT Mice

	WT	PAR-1-null
Osteoblast surface (%)*	20.0 $\pm$ 1.1	18.9 $\pm$ 0.4
Eroded surface (%)*	25.0 $\pm$ 0.7	26.5 $\pm$ 1.2
Osteoclast number/mm bone surface*	3.7 $\pm$ 0.1	4.5 $\pm$ 0.4

*All values represent mean $\pm$ SEM ($n = 4$).

PAR-4 primers was as follows: GAPDH: forward GAT GCC CCC ATG TTT GTG AT; reverse TTG CTG ACA ATC TTG AGT GAG TTG T; PAR-3: forward GCT CCA TTT GTC AGC TCC TC; reverse GGA GGG AAG GGG ACA TGT AT; PAR-4: forward GCT ACA GCC ATG CAC TCA GA; reverse AGG GCT CGG GTT TGA ATA GT. Real-time PCR was carried out on an MX3000p real-time PCR machine (Stratagene, La Jolla, CA) using 0.25 μ mol/L forward and reverse primers and Platinum Sybr Green qPCR Supermix-UDG containing ROX reference dye (Invitrogen, Carlsbad, CA). Reactions were run on the following thermal profile; hot start at 95°C for 3 minutes, then denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds, all for 40 cycles. Data were collected to the Sybr and ROX channels at the end of each extension step, and the final cycle was followed by a thermal melt step. Data collected for each sample were then analyzed using the $2^{-\Delta\Delta\text{CT}}$ method²⁵ to give expression of PAR-3 and PAR-4 (normalized to GAPDH expression in each sample) in PAR-1 null bone marrow relative to normalized PAR-3 and PAR-4 expression in WT bone marrow.

Statistical Analyses

Data were analyzed using one-tailed unpaired Student's *t*-test assuming unequal variances; *P* values <0.05 were considered significant.

Results

Histomorphometry of Uninjured Bone in PAR-1-Null and WT Mice

To determine whether there were differences in cortical bone size between PAR-1-null and WT mice at the site of surgery, cortical bone width and area were determined using transverse sections of undrilled tibiae of 12-week-old animals. The width (178 ± 8 μ m for WT mice and 174 ± 7 μ m for PAR-1-null mice) and area (0.945 ± 0.033 mm^2 for WT mice and 0.875 ± 0.032 mm^2 for PAR-1-null mice) of cortical bone showed no significant difference between PAR-1-null and WT mice. In addition, transverse sections through the fibula from 12-week-old animals were used to investigate parameters related to formation and resorption in uninjured bone. Osteoblast surface, eroded surface and osteoclast number were quantitated (Table 1); there were no significant differences for any of these parameters between WT and PAR-1-null animals.

Bone Repair in PAR-1-Null and WT Mice

In general, bone repair proceeded at a similar rate in PAR-1-null and WT mice, but differences were observed between the two genotypes at specific times and locations.

A hematoma rapidly filled the bone defect in both WT and PAR-1-null mice soon after drilling. Cells of hemopoietic appearance were present in the drill site at day 1 (not shown), but very few cells remained at day 2 (Figure 2, A and B). There were no apparent differences in blood clot formation or cellularity during the first two days between the two genotypes. In the tibiae of WT mice, a new wave of cells was observed in the drill site three days postdrilling and most of these cells were of mesenchymal appearance. At this stage, it appeared that there were fewer cells in the drill site in PAR-1-null mice than in WT mice (Figure 2, C and D). The cell density was counted in three different regions of the drill site (see Figure 1 for an illustration of the regions used for cell counts). There were about 50% fewer cells in all three areas of the drill sites of PAR-1-null mice when compared with those of WT mice (Figure 2G).

To investigate whether this difference in cell number could be attributed to differential rates of proliferation, mice were injected with [³H]thymidine 42 hours after drilling, then killed 30 hours later (ie, 3 days after drilling). Autoradiographic studies of sections from these mice indicated that the percentage of [³H]thymidine-labeled cells in the drill site was two- to fourfold lower in PAR-1-null than in WT mice (Figure 2H).

A periosteal reaction was observed adjacent to the drill site three days after drilling. The flat resting periosteal cells present before drilling became cuboidal. The periosteum was expanded dramatically from a single cell layer to multiple cell layers. However, there was no obvious difference in the periosteal reaction between WT and PAR-1-null mice at day 3 (data not shown).

By day 5, the drill site was filled with randomly arranged cells surrounded by fibrillar extracellular matrix, with no obvious difference between WT and PAR-1-null animals (Figure 2, E and F). New mineralized trabecular woven bone was present at day 7 in the drill site in both PAR-1-null and WT mice, as visualized using sections of undemineralized tissue, stained with Von Kossa stain (Figure 3, A and C). At this stage, there appeared to be no difference in the amount of new bone between the two genotypes. Two days later, however, it appeared that there was less new mineralized bone in the drill site in PAR-1-null mice than in WT mice (Figure 3, B and D). The trabecular bone area was measured in three regions of the drill site (Figure 1). The results showed that, indeed, at day 7 the values for PAR-1-null and WT animals were similar in all regions of the drill site (Figure 3E), whereas at day 9 there was significantly less bone in all regions in the PAR-1-null mice (Figure 3F). Similar results were obtained for new mineralized bone area on the periosteal surface, which was measured in a specific field of the periosteum adjacent to the drill site on the lateral surface of the tibia (Figure 1). The value obtained was less in PAR-1-null than in WT mice at day 9 (Figure 3F; $P < 0.05$), but there was no difference at day 7 (Figure 3E).

Osteoclasts appeared at day 7 in the drill sites of both PAR-1-null and WT mice, but the number of osteoclasts in the drill site was different between the two genotypes (Figure 4). There were significantly more osteoclasts in PAR-1-null mice in the lateral measurement site ($P < 0.01$) and medial site ($P < 0.05$), but the difference was not statistically significant within the bone marrow region of the drill site. In contrast, on the periosteal surface there was a trend toward fewer osteoclasts in PAR-1-null mice (0.04 ± 0.04) than in WT mice (1.93 ± 0.69 ; Figure 4) although the difference was not significant. The number of osteoclasts was increased in both the drill site and on the periosteal surface in the two genotypes by day 9, by which time there was no difference between the values for PAR-1-null and WT mice (data not shown).

During the later stages (days 14–42) of bone repair, the new bone in the medullary cavity and along the periosteal surface was resorbed, and the spaces within the cortical part of the defect filled in until the original cortical bone shape was restored (Figure 5). No morphological differences between WT and PAR-1-null mice were detected after day 9. Bone area measurements of the drill site and periosteal surface at day 14 showed no differences between the two genotypes (data not shown).

Studies on Bone Marrow Cells Isolated from PAR-1-Null and WT Mice

The cells that appear in the drill site at day 3 are largely mesenchymal in appearance, and are therefore predominantly BMSCs, which contain a population of multipotent stem cells that can differentiate into osteogenic cells.²⁶ We hypothesized that thrombin may act through PAR-1 to regulate BMSC behavior in some way that facilitates the presence of these cells in a hematoma resulting from bone injury. Thus, PAR-1 may influence the number of BMSC present in bone marrow, or it may influence proliferation, migration, or survival of BMSCs and therefore affect bone repair.

To investigate whether PAR-1-null mice have fewer stromal cells or osteoprogenitor cells in their bone marrow, both the total number of CFU-F and the number of ALP-positive CFU-F present in bone marrow from left and right femurs and tibiae were determined. There was no difference in the number of either total or ALP-positive CFU-F between WT and PAR-1-null mice (Figure 6A).

To determine whether thrombin has any effect on apoptosis induced by serum deprivation in mouse BMSCs, first-passage mouse BMSCs were investigated in apoptosis assays. The addition of thrombin (100 nmol/L) caused a significant decrease (>70%) in apoptosis in BMSCs, but the response was similar in cells isolated from WT and PAR-1-null mice (Figure 6B).

The effect of thrombin on bone marrow cell migration was investigated using freshly isolated bone marrow in Transwell plates. Nonadherent cell migration increased in the presence of thrombin, and a similar level of increase (>30%) was seen with cells from both WT and PAR-1-null mice (Figure 6C). In contrast, the migration of adherent

Figure 2. Invasion of the drill site by cells during the early stages of bone repair in PAR-1-null and WT mice. Plastic demineralized transverse Epoxy-stained sections through 2-day (**A**, **B**), 3-day (**C**, **D**), and 5-day (**E**, **F**) drill sites of WT (**A**, **C**, **E**) and PAR-1-null (**B**, **D**, **F**) mice. **Arrows** indicate bone fragments resulting from drilling. Scale bar = 100 μ m. **G:** Cell counts for three different regions of the drill site at day 3: lateral, medial and bone marrow (BM; see Figure 1). Data represent cell number in each field (mean $\pm$ SEM, $n = 5$). **H:** [3 H]thymidine-labeled cells were counted in the same regions of the drill site at day 3. Values for [3 H]thymidine-labeled cells are presented as a percentage of total cells (mean $\pm$ SEM, $n = 3$). Significant differences between values for PAR-1-null and WT mice are indicated as * ($P < 0.05$) or *** ($P < 0.001$).

Figure 3. Area of new mineralized bone in different regions of the drill site and on the periosteal surface of sections from WT (A, B) and PAR-1-null mice (C, D) at days 7 (A, C), and 9 (B, D). Arrows indicate the edges of the drill site in the medial cortex. Undemineralized transverse plastic sections were stained with Von Kossa stain. Scale bar = 400 μ m. Trabecular bone area was measured in lateral, medial and bone marrow (BM) regions of the drill site, and on the periosteal surface (POS; see Figure 1) at days 7 (E) and 9 (F). Data represent new trabecular bone area as a percentage of tissue area (mean $\pm$ SEM, $n = 5$). Significant differences between values for PAR-1-null and WT mice are indicated as * ($P < 0.05$) or *** ($P < 0.001$).

cells was not affected by the addition of thrombin in either of the genotypes (Figure 6C).

The ability of thrombin to influence proliferation of BMSCs was investigated in BrdU incorporation assays. The addition of thrombin induced a dose-dependent increase in BrdU incorporation in WT but not PAR-1-null BMSCs (Figure 6D). The stimulation of proliferation in WT BMSCs was significant at concentrations ≥ 0.01 nmol/L ($P < 0.05$).

Prothrombin and PAR Expression in Bone, Bone Marrow, and BMSCs

Polymerase chain reaction was performed to investigate expression of thrombin receptor and prothrombin mRNA

Figure 4. Osteoclasts in repair tissue of WT and PAR-1-null mice at day 7. Demineralized transverse cryosections were stained for the presence of TRAP. Multinucleate TRAP-positive cells in each field were counted manually. Data represent cell number in each field (mean $\pm$ SEM, $n = 5$). Significant differences between values for PAR-1-null and WT mice are indicated as * ($P < 0.05$) or ** ($P < 0.01$).

Figure 5. Later stages of bone repair in WT and PAR-1-null mice. Masson's trichrome-stained plastic demineralized transverse sections through the drill site of WT (A, C, E) and PAR-1-null (B, D, F) mice at days 14 (A, B), 28 (C, D), and 42 (E, F). **Arrows** indicate the edges of the drill site in the medial cortex. Scale bar = 400 μ m.

in mouse long bone, bone marrow and BMSCs from WT and PAR-1-null mice. In qualitative PCR analysis, products corresponding to PAR-1 (443 bp), PAR-3 (874 bp) and PAR-4 (476 bp) were found to be expressed in WT mouse long bone and bone marrow, whereas only PAR-1 expression was detected in WT BMSCs (Figure 7). Apart from the absence of a PAR-1 PCR product, the expression pattern was the same for PAR-1-null as for WT mice (Figure 7). In quantitative PCR analysis, no significant difference in relative expression of PAR-3 or PAR-4 was found in PAR-1-

null bone marrow compared to WT bone marrow (relative expression of PAR-3: 1.045, SEM 0.33; relative expression of PAR-4: 1.113, SEM 0.11). Analysis of relative expression of PAR-3 and PAR-4 was not carried out using long bone RNA due to the potential for contamination of long bone RNA with RNA from bone marrow.

Prothrombin mRNA expression was not detected in bone, bone marrow or BMSCs (Figure 7). PCR products corresponding to prothrombin were detected in cDNA from mouse liver (data not shown).

Figure 6. Studies on bone marrow cells. **A:** The number of CFU-F in bone marrow from both femurs and tibiae of PAR-1-null and WT mice. Colonies containing more than 20 cells were counted manually and the number of ALP-positive colonies was determined in a similar manner. The data are presented as mean $\pm$ SEM ($n = 8$ mice). **B:** The effect of thrombin on apoptosis of BMSCs isolated from PAR-1-null and WT mice. First-passage BMSCs were grown to confluence, deprived of serum for 24 hours and treated with or without thrombin (100 nmol/L) in serum-free medium for 72 hours. The percentage of apoptotic cells as a proportion of total cells was calculated. Apoptosis is expressed as a percentage of the relevant control value and presented as means $\pm$ SEM ($n = 4$ wells). **C:** Migration of bone marrow cells in response to thrombin. Freshly isolated bone marrow cells from PAR-1-null and WT mice were suspended in serum-free medium and placed in the upper chambers of Transwell plates. The lower chambers were filled with serum-free medium with or without thrombin (100 nmol/L). The cells were allowed to migrate for 5 hours, then adherent cells and nonadherent cells were counted. Data are expressed as a percentage of control values (mean $\pm$ SEM; $n = 5$). **D:** The effect of thrombin on BrdU incorporation in WT and PAR-1-null BMSCs. Subconfluent cells were deprived of serum for 24 hours. BMSCs from WT and PAR-1-null mice were treated for 24 hours with the thrombin concentrations indicated and at the same time cells were labeled with BrdU for 24 hours. The data represent mean $\pm$ SEM ($n = 4$). Significant differences between values for thrombin-treated and the relevant control values are indicated as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

Discussion

The studies presented here were initiated with the aim of determining whether thrombin, through PAR-1, influences the process of bone repair. The mouse model of bone repair used here has only recently been established,²¹ and its use in a transgenic or knockout mouse strain has not previously been reported. It provided an excellent experimental system to study bone healing in PAR-1-null mice.

The overall process of bone repair was not seriously perturbed by failure to express PAR-1, but there were significant effects on the rate of progression of certain specific events. Active thrombin is present in coagulated blood in concentrations sufficient to activate PAR-1.^{1,27} It seems likely that any role for PAR-1 in bone repair would be manifest during the early stages in the model used here, since the drill site is filled with coagulated blood immediately following drilling. Blood clot formation did not appear to be delayed by PAR-1 deficiency. This is not surprising, despite the role of thrombin in platelet activation. PAR-3 and PAR-4 mediate thrombin-induced plate-

Figure 7. Expression of thrombin receptors and prothrombin in long bone, bone marrow and BMSCs from WT and PAR-1-null mice. RT-PCR was used to examine expression of GAPDH, PAR-1, PAR-3, PAR-4 and prothrombin in RNA extracted from WT and PAR-1-null mouse long bones, bone marrow and BMSCs.

let activation in mice, and PAR-1 is not expressed in mouse platelets.¹⁴

Three days after drilling there were fewer cells in the drill site in PAR-1-null mice than in WT mice. There are several possible explanations for this observation. The cells in the drill site at day 3 are largely mesenchymal in appearance, and it is assumed that they are derived from the BMSC population and include the precursors of the osteoblasts that are abundant in the drill site and have deposited bone by day 7. PAR-1-null mice may have fewer stromal cells within their bone marrow; alternatively, thrombin may act through PAR-1 to promote survival, migration or proliferation of these cells. To investigate these possible explanations for the reduced cell number in the drill sites in PAR-1-null mice at day 3, experiments were conducted on BMSCs.

There were no differences in either total or ALP-positive colony number between PAR-1-null and WT mice. This indicates that the pre-existing stromal (including osteoprogenitor) cell number in bone marrow did not differ between the two genotypes, and therefore does not contribute to the difference in the rate of invasion of the drill site by cells of mesenchymal appearance.

The next possibility considered was that thrombin may, through PAR-1, inhibit BMSC apoptosis. Thrombin has been shown to inhibit apoptosis of myoblasts²⁸ and osteoblasts²⁴ induced by serum deprivation, although this function is not mediated by known thrombin receptors. In the current study, thrombin inhibited apoptosis of BMSCs from both PAR-1-null and WT mice, indicating that thrombin exerts a PAR-1-independent effect on apoptosis of BMSCs. The inhibition of BMSC apoptosis may be a protective effect of thrombin during the early stages of bone injury resulting in the maintenance of cell numbers essential for bone repair. However, this response to thrombin cannot explain the reduced cell number in the drill site of PAR-1-null mice.

Another possibility is that thrombin acts through PAR-1 to stimulate migration of BMSCs to the drill site. Thrombin stimulates chemotaxis of a variety of cell types. For endothelial cells, neutrophils and monocytes, this effect does not appear to require PAR-1,²⁹⁻³¹ but for vascular

smooth muscle cells and fibroblasts, the mechanism has not been thoroughly elucidated and it may involve PAR-1.^{32,33} Bone marrow cells consist of nonadherent cells (hemopoietic cells) and adherent cells consisting mostly of stromal cells (including osteoprogenitors), but also some macrophages and endothelial cells.^{34,35} Thrombin stimulated migration of nonadherent bone marrow cells from both WT and PAR-1-null mice. This suggests that mouse bone marrow hemopoietic cells migrate in response to thrombin, but that the effect is not PAR-1-mediated. In the light of these results, it seems likely that thrombin contributes to the migration of hemopoietic cells into the drill site hematoma that is detected on day one in our bone repair model. This transient migration appears to occur equally in PAR-1-null and WT mice, in keeping with the PAR-1-independent response observed *in vitro*. In contrast, thrombin had no effect on migration of adherent mouse bone marrow cells isolated from either WT or PAR-1-null mice. These results suggest that thrombin is not responsible for recruitment of BMSC to the site of injury during bone damage; thus, differential migration rates are unlikely to account for differences in cell number in drill sites of WT and PAR-1-null mice.

The remaining possibility is that thrombin, through PAR-1, stimulates BMSC proliferation. Previous studies have shown that thrombin is a mitogen for many cell types, including vascular smooth muscle cells,^{36,37} fibroblasts,^{38,39} endothelial cells,⁴⁰ mesangial cells⁴¹ and osteoblasts.⁴ In many of these cells, proliferation is known to be mediated by PAR-1 and in osteoblasts, in particular, we have recently confirmed that proliferation is mediated by PAR-1.⁵ In the current study, the addition of thrombin led to an increase in BrdU incorporation in BMSCs from WT mice but not in BMSCs from PAR-1-null mice. This result indicates that thrombin stimulates BMSC proliferation and that PAR-1 is necessary for the response. This effect is likely to provide an explanation for the observation that there are more cells in the drill site in WT mice than in PAR-1-null mice three days after bone injury. This conclusion is supported by the observation that a lower percentage of the cells in the drill site at day 3 were labeled with [³H]thymidine in PAR-1-null mice than in WT mice.

The next obvious difference between PAR-1-null and WT mouse bone repair tissue was observed at day 7, when there were more osteoclasts in drill sites of PAR-1-null mice than WT mice. Thrombin was shown many years ago to stimulate osteoclastic resorption in organ culture,^{6,7} but it is not yet known whether this effect is due to stimulation of osteoclast differentiation or activity (or both); nor is it known whether the effect is mediated by PAR-1. The results presented here do not really shed any light on this question, but they do suggest that PAR-1 is not necessary for osteoclast differentiation during bone healing. On the contrary, there appear to be abnormally high levels of stimulation of osteoclast differentiation during bone repair in PAR-1-null mice. Osteoclasts are derived from hemopoietic precursors under the influence of essential factors provided by other cell types, including cells of the osteoblast lineage. It is possible that one of these cell types, not present in the organ culture systems

referred to above, exerts a PAR-1-mediated inhibitory effect on osteoclast differentiation. Alternatively, the bone produced in the early response to injury may be recognized as defective, thus leading to premature removal by osteoclasts. Further investigation will be required to elucidate the mechanisms leading to enhanced osteoclast numbers.

New mineralized bone appeared at day 7 in the drill site of both PAR-1-null and WT mice. Similar amounts of bone were present in the two genotypes. Surprisingly, at day 9, less new mineralized bone was present in the drill site in PAR-1-null mice. However, there were more osteoclasts in PAR-1-null mice at day 7, so it is most likely that elevated bone resorption in the drill site from day 7, rather than less bone formation, leads to the decreased trabecular bone area at day 9 in PAR-1-null mice. At day 9, there was less bone per tissue area not only in the drill site, but also on the periosteal surface in PAR-1-null mice than in WT mice. Since the number of osteoclasts on the periosteal surface was no different between WT and PAR-1-null mice either at day 7 or day 9, the reason for the decreased periosteal bone in PAR-1-null mice could be reduced bone formation rather than elevated bone resorption. This suggests that the mechanism of bone modeling on the periosteal surface is different from in the drill site, and that PAR-1 affects different osteoprogenitor cells (periosteal or bone marrow) differently.

Another interesting aspect of the observations presented here is that there was an effect of PAR-1 deficiency on periosteal tissue at all. The experiments were initiated primarily because it was certain that there would be active thrombin in the drill site hematoma in the early stages following injury, but it was not clear that this would be the case for the periosteum. The periosteal reaction probably occurs as part of an inflammatory reaction radiating from the drill site into adjacent tissues. This could explain the presence of active thrombin in the adjacent periosteum; thrombin not only facilitates the inflammatory response but it is also likely to be present in active form in tissues undergoing inflammation due to the various associated vascular events (including increased permeability to plasma proteins). Another possible source of active thrombin, present in the periosteum adjacent to the drill site, is prothrombin expressed and activated by skeletal muscle.^{42,43}

There were no differences observed between PAR-1-null and WT mice after day 9, and bone repair was complete at day 42 in both genotypes. The lack of effect of PAR-1 deficiency in the later stages could be explained by thrombin activity only being present during the early stages of bone repair. This explanation seems likely, since in the current study, prothrombin expression was not detected by RT-PCR in bone or bone marrow. Further support for this conclusion is provided by the observation that for a number of histomorphometric parameters in uninjured bone, there were no detectable differences between WT and PAR-1-null animals. Thus, it appears that thrombin does not exist in the normal bone environment, suggesting that it only plays roles during early bone healing or other conditions involving disruption of vascular integrity. Many factors are involved in the

normal progression of bone healing, and factors other than thrombin are likely to predominate at the later stages. It is possible that other ligands of PAR-1 may be present in bone, but it is unlikely that they are responsible for any of the observations made in the current study. Trypsin and factor Xa have been shown to activate PAR-1, but at much higher concentrations than thrombin.² Like thrombin, factor Xa is likely to be present in bone in conditions involving loss of vascular integrity, but trypsin is unlikely ever to be present in bone.

The possibility that thrombin, through receptors other than PAR-1, might be important for bone healing cannot be excluded. For example, in the current study PAR-3 and PAR-4 expression were detected in mouse bone and bone marrow, and we have previously demonstrated that mouse osteoblasts express PAR-4. Thus thrombin may influence bone repair through any or all of PAR-1, PAR-3, and PAR-4. It is interesting to note that expression of these other thrombin receptors appears to be unaffected by the absence of PAR-1.

In conclusion, the drill site was populated by BMSCs more slowly in PAR-1-null mice than WT mice, an effect that can be explained by the PAR-1-dependent effect of thrombin on BMSC proliferation. The reduction in bone area in PAR-1-null mice at day 9 in the drill site may be a result of the increased number of osteoclasts present at day 7. However, the decrease in bone area on the periosteal surface in PAR-1-null mice is likely to result from less bone formation. These observations indicate that PAR-1-mediated responses to thrombin play a role in early bone repair.

Acknowledgments

We thank Dr. S.R. Coughlin (University of California at San Francisco) for providing the PAR-1-null mice, Ms. S. Toulson for assistance with animal care, and Dr. T.D. Hewitson (Royal Melbourne Hospital, Australia) for helping with autoradiography.

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