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Thrombin mediated migration of osteogenic cells

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Abstract

Given that thrombin is ubiquitously expressed at sites of vascular injury, and that osteogenic cells express receptors for thrombin, we questioned whether thrombin could attract osteogenic cells to a bony wound. Using a scratch wound assay, thrombin stimulated a significant increase in migration of osteogenic cultures of primary marrow cells. This effect was dependent on thrombin proteolytic activity; however, thrombin was unable to stimulate the migration of a more differentiated marrow-derived osteogenic cell line. To better understand the role of thrombin in osteoprogenitor migration, we developed an osteoprogenitor migration assay that combines a modified Boyden chamber with a bone nodule assay. Primary cells that migrated through the transwell filter in the presence of thrombin formed significantly more bone nodules compared to the condition without thrombin. This was not due to proliferation or differentiation effects of thrombin. In contrast, thrombin was unable to stimulate an increase in the number of nodules for the more differentiated osteogenic cell line. Thus, our results suggest that thrombin exhibits differential motogenic effects on osteogenic cells depending on their differentiation state. The cell migration/bone nodule assay described here is the first assay that can be specifically used to examine the effects of factors on the migration of osteoprogenitor cells, particularly those derived from primary populations.

Keywords: Thrombin; Osteogenic cells; Migration; Osteoprogenitors; Stroma

Introduction

During bony wound healing, osteogenic cell migration from the bone marrow compartment to the injury site is regulated, in part, by factors released by platelets and macrophages such as platelet-derived growth factor-BB (PDGF-BB) [29] and transforming growth factor beta 1 (TGFβ1) [5]. These factors may diffuse into the bone marrow, where populations of both committed osteogenic cells and osteoprogenitors reside [7]. In addition to PDGF-BB and TGF-β1, cell migration during wound healing may be stimulated by thrombin, which is generated from prothrombin during the blood coagulation cascade [10,12,20].

Thrombin is a multifunctional protein that is ubiquitously expressed at sites of vascular injury. It cleaves fibrinogen, via its proteolytic activity, to polymerize fibrin and activates clotting cofactors such as factor V and VIII, which accelerate the coagulation process, and factor XIII which helps stabilize the blood clot by cross-linking fibrin [38]. In addition to serving an essential role in forming the provisional fibrin matrix, through which cells migrate during wound healing, thrombin has been demonstrated to interact

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with specific cell surface receptors [32,53] and this may stimulate a variety of events including platelet activation.

Since the first putative receptor for thrombin, protease activated receptor-1 (PAR-1), was discovered over a decade ago [53], three additional protease activated receptors have been identified. Thrombin activates intracellular signaling pathways by interacting with three of these transmembrane domain G-protein-coupled receptors (PAR-1, PAR-3, PAR-4) [32]. These receptors become activated by proteolytic cleavage of an extracellular domain thereby exposing a new amino terminus that acts as a tethered ligand, docking intramolecularly with the body of the receptor [53].

Through interacting with protease activated receptors, thrombin can stimulate proliferation [13,14,26,28,45,51] and migration [10,15,17,23,35] of a variety of cell types. With respect to osteogenic cells, which express PAR-1 [2,3], PAR-2 [1], and PAR-4 [36], thrombin has been demonstrated to stimulate the proliferation of both primary, calvarial, and long bone-derived, osteogenic cells [3] and osteosarcoma-derived cell lines [48,52]. Thrombin has also been demonstrated to stimulate the migration of highly metastatic osteosarcoma cells [39]; however, the ability of thrombin to stimulate the migration of primary derived osteogenic cells had not been determined.

In this study, a 2-D scratch wound model was used to examine the effects of thrombin on the proliferation and migration of rat bone marrow-derived adherent cells under osteogenic culture conditions. Since primary bone marrow cells contain a heterogeneous population of cells, a bone marrow-derived osteogenic cell line was used to provide evidence that the observed effects applied to an osteogenic population. In addition, we combined a modified Boyden chamber with a bone nodule-forming assay to determine if thrombin could stimulate the migration of osteoprogenitor cells, and thereby increase the amount of bone formed by the migrated cells.

Methods

Tissue culture polystyrene 6-well, 12-well, and 96-well flat bottom plates were purchased from Corning Glass Works and Falcon® 75-cm^2 flasks, 35-mm dishes, and 50-ml tubes from BD Biosciences. Alpha-minimal essential medium (α-MEM), fetal bovine serum (FBS), gentamicin, and phosphate-buffered saline (PBS)-based enzyme free cell dissociation buffer were obtained from Invitrogen. Transwell filters having 8-μm pores that fit into 6-well plates were obtained from Fisher Scientific. Transwell filters with 20-cm^2 surface area and AS1 single well separation chambers were purchased from Neuro Probe Inc. Penicillin G, amphotericin, dexamethasone, L-ascorbic acid, sodium-β-glycerophosphate, bovine serum albumin (BSA), PDGF-BB, and mitomycin-C were obtained from Sigma. Human alpha thrombin (3181 NIH U/mg) was obtained from Enzyme Research Labs, and S-2238 thrombin chromogenic substrate was obtained from DiaPhara Inc. Thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (PPACK) was obtained from Biomol, FITC-phalloidin from Molecular Probes, and TGF-β1 from Chemicon.

Primary cell isolation and culture

Rat bone marrow cells were isolated as previously described [33]. The maintenance and use of animals were in accordance with the Canadian Council of Animal Care Guidelines. Fifteen milliliters of the primary cell suspension was cultured in T75 flasks in medium containing α-MEM supplemented with 15% (v/v) FBS. The media were changed on day three, and on day four, the cells were released from the flasks using enzyme free cell dissociation buffer, centrifuged, resuspended in culture media, placed through a 70-μm cell strainer and counted using a hemocytometer. Dexamethasone was added at a concentration of 10^{-8} M for all experiments involving primary cells.

RBMC/D8 cell line culture

The clonal RBMC/D8 cell line used in this study was generated from the isolation of single colonies of spontaneous immortalized primary rat bone marrow cells, grown in the presence of 3 ng/ml fibroblast growth factor-2 (FGF-2) and 10^{-7} M dexamethasone [27]. The RBMC-D8 clone was selected based on its ability to differentiate in osteoblastic cells that form large numbers of mineralized bone nodules within 2 weeks in the presence of 5 mM sodium-β-glycerophosphate. Also, unlike the parental cells, RBMC-D8 cells do not require FGF-2 or dexamethasone to form mineralized nodules (Jheon, Bansal, Sodek, Pitaru, and Cheifetz, unpublished results). Cells were maintained by subculturing at ~75% confluency into T75 flasks in medium containing α-MEM supplemented with 15% (v/v) FBS. Cells were subcultured using the same methods used for the primary cells without dexamethasone. Cell line cultures were maintained for no longer than 8 weeks after recovery from frozen stocks.

Bone nodule assay

The bone nodule assay [11] consisted of culturing cells for 14–17 days with osteogenic culture media which contained α-MEM supplemented with 15% (v/v) FBS, 50 μg/ml ascorbic acid, and 5 mM sodium-β-glycerophosphate and 10^{-8} M dexamethasone together with antibiotics and fungizone. Media were changed every 2–3 days. To determine if the cell line could produce mineralized nodules, cells were transferred into 6-well plates at 10^5 cells per well in osteogenic media. Nodules were observed by electron microscopy as previously described [19,25,33]. To quantify the number of nodules produced in culture, 10^4 cells were placed in wells of 6-well plates and allowed to adhere in 15% FBS for 24 h. The cultures were maintained for 13–16

days, at which point the cultures were re-fed with medium containing 9 mg/ml tetracycline HCl (Sigma), incubated for 24 h, fixed in Karnovsky's fixative, and then prepared for analysis [37]. A ChemiImager 5500 (Alpha Innotech Corporation, San Leandro, CA, USA) was used to visualize the tetracycline-labeled cultures. To determine if a pulse of thrombin could stimulate nodule formation, wells containing adhered cells were rinsed twice with PBS and incubated for 24 h in 1% BSA alone or in 1% BSA supplemented with 1 U/ml thrombin. Wells were rinsed with PBS and replaced with osteogenic media.

Analysis of mRNA expression

Total RNA extraction, cDNA synthesis, Northern transfer of RNA followed by hybridization with probes were performed as previously described [49,50]. Briefly, rat bone marrow cells were cultured for 4 days in α-MEM supplemented with 15% FBS and 10^{-8} M dexamethasone. Total RNA was extracted with TRIZOL (Invitrogen) after 24-h incubation in α-MEM with 1% BSA, 1% BSA with 1 U/ml thrombin, or 10 ng/ml TGF-β1. To obtain probes, RNA from 1% BSA-treated cells was primed with random hexamers, followed by first strand cDNA synthesis with Superscript III (Invitrogen) at 55°C for 1 h. PAR1 and matrix metalloproteinase-2 (MMP-2) as probes for Northern hybridization were obtained by Expand High-fidelity PCR system (Roche) with primer pairs as previously described [30,47]. The PCR products using PAR-1 (731 bp)- and MMP-2 (591 bp)-specific primers showed exclusive hits with PAR-1 and MMP-2 by BLAST search against both non-redundant NCBI and rat genome databases (not shown). Hybridization was performed at 65°C for 22 h with QuikHyb (Stratagene) containing 1 mg/ml Salmon Sperm DNA (Invitrogen), followed by washing twice at RT with 2× SSC, 0.1% SDS, 2× at 65°C with 2× SSC, 0.1% SDS and 2× at 65°C with 0.1× SSC, 1% SDS. Washed membranes were exposed to Kodak Biomax film at −86°C for 1 h and 20 h to confirm the linearity of the signals.

Scratch wound migration assay

Once the cells in the 6-well plates were confluent, monolayers in each well were scratched with sterile-plastic pipette tips and rinsed twice with PBS before incubating the cells in α-MEM supplemented with 1% BSA and one of a variety of factors. To quantify a leading front of cell migration, images were taken at 0 h and 24 h later, using a microscope equipped with a digital camera, followed by analysis with image analysis software (Image Pro Plus 4.1). To ensure images were taken of the same area at both time points, a straight horizontal line (perpendicular to the scratch wounds) was made on the underside of each well, and this was positioned at the bottom of each field before acquiring the images. The negative control consisted of α-MEM containing 1% BSA and the positive control consisted of α-MEM with 15% FBS. Thrombin was added at concentrations of 0.5, 1.0, 2.0, and 5.0 U/ml. To determine if the effect of thrombin on cell migration was mediated via thrombin proteolytic activity, thrombin was pre-incubated with 200 nmol of the thrombin inhibitor PPACK for 30 min at room temperature. PPACK without thrombin served as a negative control. In some experiments, cell proliferation was inhibited by pretreating the primary cells and cell line with 20 μg/ml and 5 μg/ml of mitomycin-C, respectively, for 30 min at 37°C prior to 'scratching'. The effective mitomycin-C concentrations were determined using a BrdU cell proliferation assay. After treatment with mitomycin-C, cells were washed three times with PBS, covered with media containing 15% FBS and incubated for at least 1 h prior to performing the scratch assay.

Cytochemical analysis of actin filaments

Cells were seeded into 35-mm dishes for 3 h in medium containing 15% FBS. After rinsing each well with PBS three times, cells were incubated in either 1% BSA or 1% BSA with 1 U/ml thrombin. Cultures were fixed in a 2% formaldehyde/2% gluteraldehyde solution after 1, 24, and 48 h. The polymerization of filamentous actin (F-actin) was determined by staining the cells with FITC-conjugated phalliodin in PBS (1:200) after treating the cells with 0.1% Triton X-100 in PBS for 90 s. All samples were examined with a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY) equipped with a 40× Axiovert water lens.

Thrombin activity assay

To determine the activity of thrombin, a 70-μl sample of media from the cell cultures containing thrombin was collected at various time points and added to 30 μl of 1 mmol S-2238 thrombin chromogenic substrate within wells of a 96-well plate. A Versamax microplate reader (Molecular Devices) was used at 37°C at a wavelength of 40 nm to determine the slope of the absorbance versus time curve, over a 2-min interval. Experiments were also performed using media from wells devoid of cells and wells that contained fixed cells. The thrombin chromogenic substrate S-2238 was also added to media from cell cultures that did not contain thrombin to determine if the substrate could interact with factors produced by the cells. To ensure that thrombin treated with the thrombin inhibitor PPACK was devoid of catalytic activity, 1 U/ml thrombin in 1% BSA was incubated with PPACK for 30 min at room temperature. The thrombin–PPACK complexes were combined with the S-2238 substrate and examined with a microplate reader as described above. Untreated thrombin (1 U/ml) in 1% BSA and 1% BSA alone served as positive and negative controls, respectively.

Cell migration/bone nodule assay

Briefly, 50,000 cells were added to the top compartment of transwell filters that were placed into the wells of 6-well plates. Cells were allowed to adhere for 10 h in the presence of α-MEM supplemented with 15% FBS after which the wells were rinsed with PBS. Following the addition of α-MEM with 1% BSA or 1% BSA with 1 U/ml of thrombin, to the upper and lower compartments, the cells were incubated for 24 h and then the cells on top of the filter were removed with a cotton swab. After rinsing the upper and lower compartments three times with PBS, osteogenic media were added to both compartments. The cells were incubated for an additional 14–17 days with osteogenic media changes every 2 or 3 days. Areas containing mineralized regions were counted as described above.

To determine the numbers of cells on the underside of the filters prior to switching to osteogenic media, the tops of some filters were scraped and then whole filters were stained with toluidine blue. Cells from thirty random 0.01 cm^2 light microscope fields were counted for each condition (representing 6.7% of the filter surface area). To determine if thrombin could influence osteopontin content in migrating cells, migrant and non-migrant populations were separated using a large modified Boyden chamber (AS1 single separation chamber, Neuroprobe Inc.) to facilitate enough protein extraction for Western blots. Migrant and non-migrant cells were defined as cells that had migrated to the underside of the transwell filter, or had remained on the upper surface of the filter, respectively. Briefly, polycarbonate filters (8 μm pore size; Neuroprobe Inc.) having a surface area of 20 cm^2 were washed and coated with Vitrogen (Sigma) as previously described [59]. The filters were housed within single separation chambers. After incubating $2.5–3.0 \times 10^6$ cells from day 4 primary osteogenic cultures on the upper surface of the filters in the presence of 15% FBS for 10 h, wells were rinsed three times with PBS followed by a 24-h incubation with either 1% BSA or 1% BSA with 1 U/ml thrombin in both the top and bottom chambers. Protein was extracted from migrant and non-migrant cells and osteopontin content was determined with Western blots using a monoclonal mouse anti-rat osteopontin antibody (MPIII-B101) and normalized for cell number with actin as previously described [58].

Statistical analysis

For multiple comparisons, analysis of variance was performed with the Tukey's HSD test. For single comparisons, a paired Student's *t* test was used. Standard deviations are presented in the text. Error bars in bar graphs represent the 95% confidence interval, and statistical significance is denoted by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

Thrombin activity stimulates the migration of primary bone marrow stroma

Thrombin is expressed at sites of injury, including bone, and plays a critical role in migration of a wide range of cell types. To test the ability of thrombin to stimulate osteogenic cell migration, we performed a scratch wound assay on primary rat bone marrow stromal cells. In fact, thrombin stimulated a 2.0 ± 0.3-fold ($P = 0.003$) increase in the leading front of migration for the primary rat bone marrow stromal cells (Fig. 1A). Titration of thrombin demonstrated increasing migration up to 1 U/ml thrombin, with no substantial increase beyond this concentration (data not shown).

The increased migration was dependent on thrombin proteolytic activity. Thrombin was treated with 200 nM PPACK, a specific inhibitor of α-thrombin proteolytic activity, for 30 min. Analysis of proteolytic cleavage of

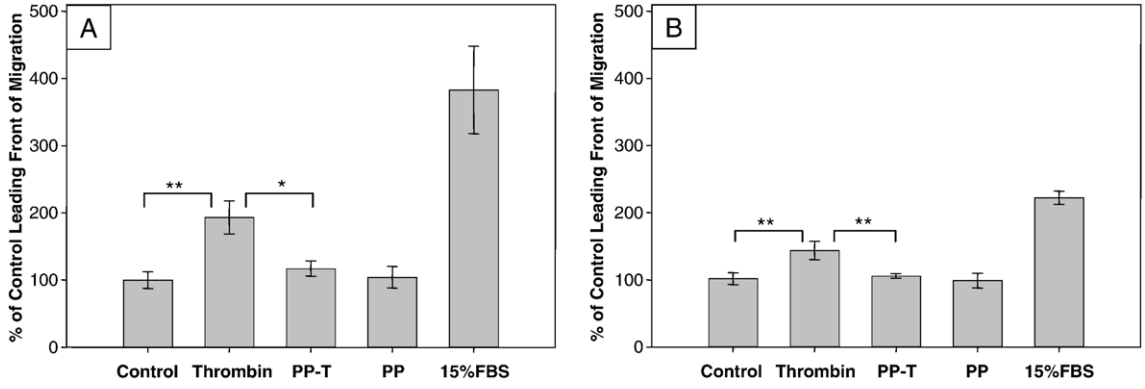

Fig. 1. Thrombin (1 U/ml) induces cell spreading of primary bone marrow stromal cells and the RBMC/D8 osteogenic cell line in a scratch wound assay. Blocking the active site of thrombin by PPACK (PP-T) significantly inhibited the response for both (A) the primary bone marrow stromal cells ($n = 7$) and (B) the RBMC/D8 osteogenic cell line ($n = 3$). No statistical difference was found between PPACK-treated thrombin (PP-T) and PPACK-alone (PP) conditions. All measurements of cell spreading (change of the leading front of cells) were normalized (by percentage) with the leading front of negative control cultures (1% BSA, control).

the thrombin chromogenic substrate S-2238 demonstrated that PPACK-treated thrombin had less than 0.2 ± 0.1% ($P < 0.001$) of its original activity, while the activity of thrombin without PPACK treatment did not substantially deviate from its original activity when incubated under identical conditions (data not shown). Over 80% of the migration induced by thrombin was inhibited when PPACK-treated thrombin was used in the scratch wound assay of primary osteogenic cells ($P = 0.032$), whereas PPACK alone did not induce a response (Fig. 1A).

Primary stromal cells are a heterogeneous population, including multi-lineage progenitors as well as committed progenitors to various lineages including bone. To better understand the role of thrombin in the migration of committed osteoprogenitors, we used an osteogenic cell line (RBMC/D8), obtained from spontaneously transformed rat bone marrow cells. These cells express bone matrix proteins, collagen, bone sialoprotein, osteopontin, osteoclacin, and SPARC/osteonectin, together with alkaline phosphatase in a temporal pattern (Jheon et al., in preparation), similar to that observed previously with freshly-isolated rat bone marrow cells [56]. However, RBMC-D8 cells do not require dexamethasone to form bone nodules in culture and thus could be considered as a more mature population of osteogenic cells. This is supported by the early expression of bone sialoprotein and the more rapid formation of bone nodules. As shown in Fig. 2, RBMC/D8 cells form bone nodules when grown under osteogenic conditions for 7–10 days. Examination of the culture dish surface by scanning electron microscopy after 14 days revealed the presence of a cement line matrix (Fig. 2A), which is the first matrix deposited by differentiating osteogenic cells during de novo bone formation [18], to which collagen fibers had anchored and subsequently mineralized (Fig. 2B). We found a tenfold increase in the frequency of clonogenic progenitors capable of forming bone nodules (colony forming unit-osteoblast, CFU-O) in the RBMC/D8 cell line compared to rat primary stromal cells. CFU-O frequencies in three independent experiments averaged a frequency of 1:162 and 1:1589 cells, based on input cell numbers, for the RBMC/D8 cell line and primary stromal cells, respectively.

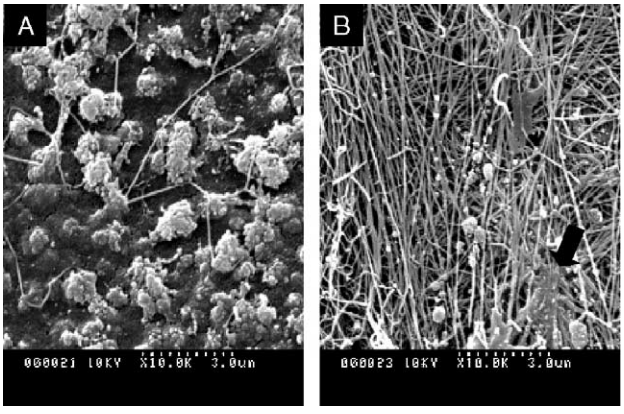

Fig. 2. Bone nodule formation by the bone marrow-derived RBMC/D8 osteogenic cell line. Scanning electron micrographs show that the RBMC/D8 osteogenic cell line produces many of the hallmarks of de novo bone formation including (A) a mineralized cement line matrix and (B) collagen which anchors to the cement line matrix and becomes mineralized. (Black arrow points to an area of mineralized collagen.)

Consistent with the increased migration stimulated by thrombin in primary cells, thrombin also stimulated an increase (1.3 ± 0.2-fold, $P = 0.040$) in the leading front of migration in the RBMC/D8 osteogenic cell line (Fig. 1B), although to a much lesser extent than observed in primary cells. Furthermore, this enhanced migration was also dependent upon thrombin activity, demonstrated by PPACK inhibition of thrombin-induced migration ($P = 0.004$) (Fig. 1B).

Given that the effects of thrombin were mediated via thrombin proteolytic activity, we were interested in determining if the activity of thrombin changed during the course of the scratch wound assay and if this was cell mediated. Fig. 3 shows how the activity of thrombin decreased in the presence of both cell types, but no significant decrease in thrombin activity was observed within empty petri dishes or dishes containing fixed osteogenic cell line-derived cells. After 24 h, approximately 20% of the initial thrombin activity, as measured by cleavage of the S-2238 thrombin chromogenic substrate, remained in the supernatant of the primary cells, whereas the thrombin activity became undetectable after 18.5 h incubation with RBMC/D8 cells. The coefficient of determination (R^2) was 0.84 ($P < 0.001$) for the primary cells and 0.89 ($P < 0.001$) for the cell line. When thrombin-deficient supernatant of the primary cells was combined with the S-2238, no change in absorption was observed, demonstrating that there were no significant interactions between media, or cellular components and S-2238.

To determine if thrombin treatment simply induced proliferation or migration of existing cells, cells were treated with mitomycin-C to inhibit proliferation. Treatment with 20 μg/ml of mitomycin-C for 30 min reduced the primary leading front of thrombin-induced migration by approximately 30% (not shown). Despite inhibition of proliferation, thrombin (in 1% BSA) was able to stimulate a 213 ± 66% ($P = 0.042$) increase in primary cell coverage of the wounded area compared to the mitomycin-C negative control (Fig. 4A). This response decreased to 111 ± 16% ($P = 0.034$) stimulation upon treatment with the PPACK–thrombin inhibitor. Furthermore, compared to the negative control, 10 ng/ml PDGF-BB and 15% FBS stimulated 296 ± 37% ($P = 0.004$) and 602 ± 144% ($P < 0.001$) more cell coverage, respectively. In contrast, thrombin did not stimulate a response in the RBMC/D8 osteogenic cell line (Fig. 4B); however, PDGF-BB and 15% FBS stimulated a migration response of 164 ± 42% ($P = 0.004$) and 212 ± 12% ($P < 0.001$) compared to the mitomycin-C negative control.

Actin filaments demonstrate distinct changes in migrating cells. Thus, we examined the effects of thrombin on actin polymerization in primary bone marrow stromal cells and the RBMC/D8 osteogenic cell line. Although actin

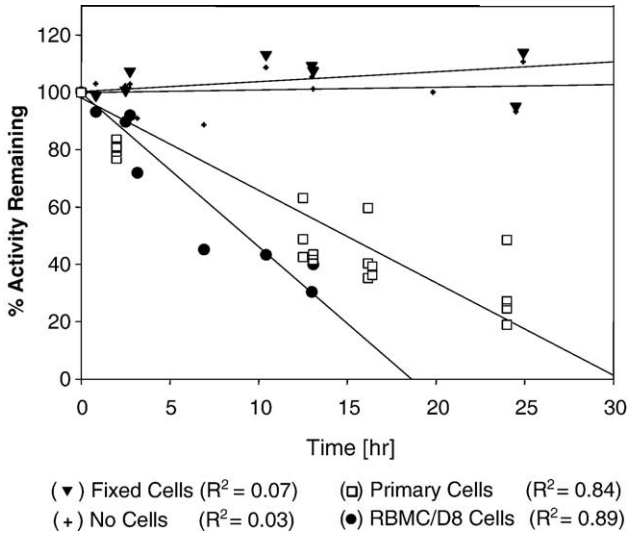

Fig. 3. Thrombin activity rapidly declines in cell culture. Thrombin activity was measured by cleavage of the S-2238 thrombin chromogenic substrate versus time (in hours, h). Thrombin activity decreased quickly in the presence of both (□) the primary bone marrow stromal cells and (●) the RBMC/D8 osteogenic cell line (n = 3). However, the activity did not change when incubated with (▼) fixed cell line cultures or with (+) no cells. The slopes of the regression lines were not found to be significantly different from the horizontal.

filament staining was similar in primary cells treated with either 1% BSA or 1% BSA with thrombin for 1 h (Figs. 5A,B), cells that were treated with thrombin displayed increased cell spreading and staining intensity after 24 h (Figs. 5C,D). After 48 h (Figs. 5E,F), unlike cells treated with only 1% BSA, distinct actin filaments were detected in the primary stromal cells that were treated with thrombin. In contrast to the primary cells, the RBMC/D8 osteogenic cell line (Figs. 5G–L) displayed distinct actin filaments after 24 h in 1% BSA and 1% BSA with thrombin (Figs. 5I,J), yet thrombin treatment did not induce any detectable differences at any of the time points.

Thrombin stimulates the migration of primary osteoprogenitors

Given that there is a lack of specific markers for the osteogenic lineage, especially for osteoprogenitors, we created a new assay to examine the migration of osteoprogenitors based on counting the number of bone nodules formed after the cells had migrated (Fig. 6). The number of bone nodules formed in culture is believed to correspond to the number of osteoprogenitors in culture. This assay required the isolation of the migrated cells and further culture of these cells in the presence of osteogenic supplements. To accomplish this, cells were first cultured in the presence or absence of thrombin within modified Boyden chambers. After isolating the migrating population, the migrated cells were cultured in media containing osteogenic supplements (as described above) and the number of bone nodules that formed was counted. As evidenced from tetracycline staining of mineralized nodules, the primary cells that migrated through a transwell filter in response to a pulse of thrombin produced 50% (P = 0.040, n = 7) more bone nodules compared to cells that were cultured in the absence of thrombin (Fig. 7A). This increase in nodule number was associated with a 21 ± 14% (P = 0.250, n = 6) increase in cell number detected on the underside of filters treated with a pulse of thrombin compared to filters treated with a pulse of 1% BSA alone (not shown). When dexamethasone was omitted from the osteogenic media, significantly fewer nodules formed on the underside of the filters, and a pulse of thrombin had no effect on nodule formation (P = 0.998, n = 3). The complete removal of the non-migrated cells from the upper portion of the filters, after the 10-h incubation in 15% FBS, was verified by scraping the top and/or bottom of selected filters followed by toluidine blue staining and observation by light microscopy.

To determine if the thrombin-stimulated increase in cell number could account for the increased number of bone

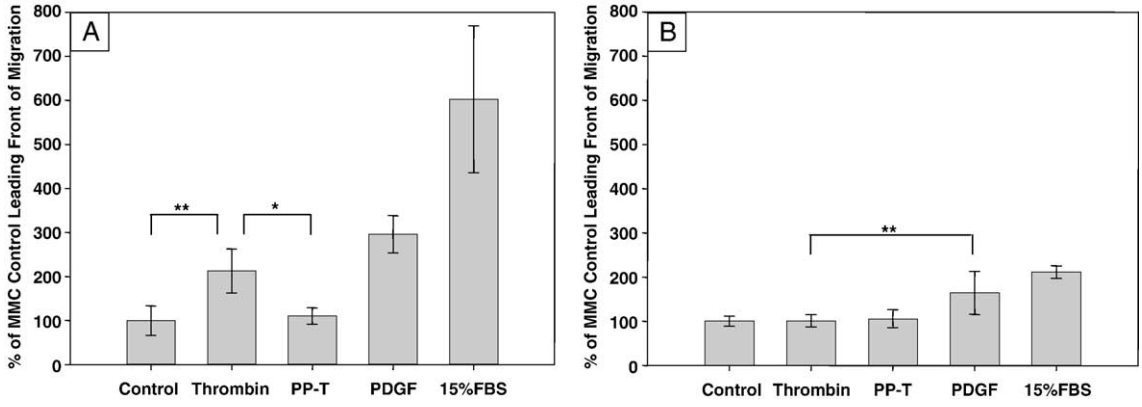

Fig. 4. The effect of mitomycin-C blocked proliferation upon thrombin-induced cell spreading in the scratch wound assay. (A) In the absence of proliferation, 1 U/ml thrombin, 10 ng/ml PDGF-BB (PDGF), and media containing 15% FBS stimulated the migration of the primary bone marrow stromal cells. The lack of response from PPACK-treated thrombin (PP-T) indicates that thrombin proteolytic activity is required to stimulate migration of the primary bone marrow stromal cells. In contrast, (B) although PDGF-BB and 15% FBS stimulated the migration of the RBMC/D8 osteogenic cell line, thrombin had no effect. All experimental conditions were normalized with mitomycin-C-treated negative control (n = 3–7).

nodules, cells were seeded into 12-well plates representing twice the number of cells counted on the bottom of the transwell filters treated with 1% BSA or 1% BSA with thrombin. A 21% increase in cell number stimulated only 0.3 ± 0.6 more nodules compared to the condition with fewer cells ($P = 0.670$). Nodules formed on the bottom of transwell filters (Fig. 8) displayed many of the hallmarks of de novo bone formation including (Fig. 8A) a cement line matrix and (Figs. 8A,B) an overlying collagenous matrix. When the cell line was employed in the cell migration/bone nodule assay, 42 ± 20 nodules were produced in the presence of thrombin compared to 41 ± 17 nodules in the absence of thrombin ($P = 0.588$, $n = 4$).

Osteopontin expression had been shown to be upregulated in migrating stromal cells [58]. Moreover, since thrombin can cleave osteopontin and potentiate its effects as a chemoattractant [42,43], we next determined if the migration of the primary stromal cells in response to thrombin was mediated through osteopontin. Both migrant and non-migrant cells expressed intracellular osteopontin (Fig. 7B) as previously described [58]. Specifically, migrant cells contained over fivefold higher levels of osteopontin than non-migrant cells in 1% BSA. Addition of 1 U/ml thrombin stimulated over an eightfold increase in intracellular osteopontin levels in migrant cells compared to non-migrant cells, and a twofold increase in osteopontin levels in migrant cells compared to the migrant cells treated with 1% BSA, suggesting that osteopontin may mediate the migration of osteogenic cells in response to thrombin.

Thrombin upregulates the PAR-1 transcript

To determine if the effects of thrombin on the migration of the primary cells and the osteogenic cell line were mediated via the PAR-1 receptor, Northern and semi-quantitative reverse transcriptase-PCR (RT-PCR) analysis were performed. Northern analysis showed that the PAR-1 transcript was expressed constitutively in bone marrow stromal cells before and after a 24-h incubation in 1% BSA (Fig. 9) and was upregulated after a 24-h

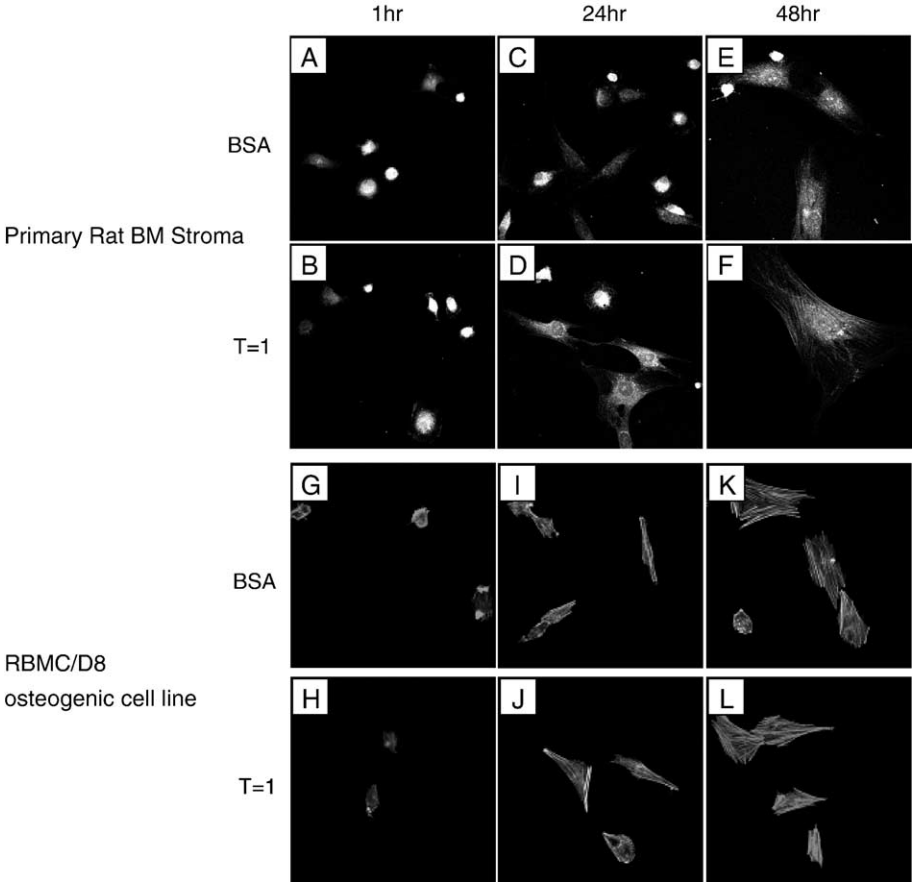

Fig. 5. Thrombin stimulates actin polymerization. Representative images of FITC-phalloidin stained F-actin from (A–F) primary bone marrow stromal cells ($n = 3$) and from (G–L) the RBMC/D8 osteogenic cell line ($n = 3$). (A,B) Primary stromal cells began to spread and extended cellular processes after 1 h of incubation in 1% BSA and in 1% BSA containing 1 U/ml thrombin ($T = 1$). (C,D) After 24 h, the thrombin-treated primary bone marrow stromal cells were more spread and displayed more intense staining compared to cells treated with 1% BSA alone. (E,F) After 48 h, primary bone marrow stromal cells treated with thrombin expressed long, densely stained, and highly oriented filaments. In contrast, (I,J) the RBMC/D8 cell line expressed long densely stained and highly oriented filaments after 24 h in both the presence and absence of thrombin. After 48 h (K,L), the cells treated with 1% BSA or with 1% BSA containing thrombin were highly spread and the presence of oriented filaments was more apparent; however, no significant differences were observed between these groups.

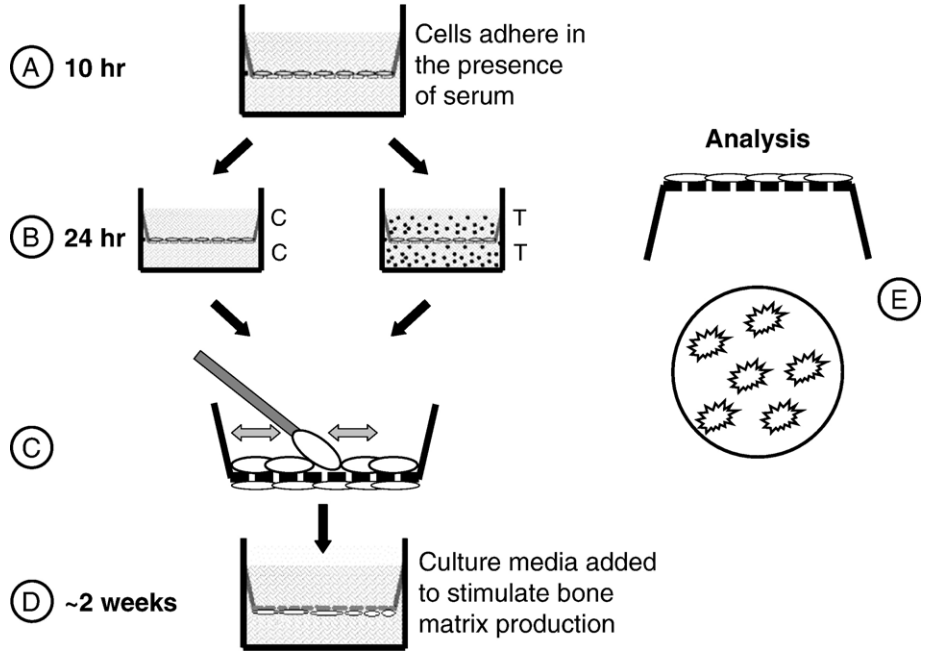

Fig. 6. Combined cell migration and bone nodule assay. (A) Cells were first allowed to adhere to the upper surface of modified Boyden chambers for 10 h in the presence of α-MEM supplemented with 15% serum. (B) After rinsing the chambers 2–3× with PBS, the cells were incubated in the presence α-MEM with either 1% BSA or 1% BSA with 1 U/ml thrombin for 24 h. (C) The non-migrated cells were removed by a scraping instrument from the upper surface of the chamber leaving only the migrated cells growing now on the bottom surface. (D) The migrated cells were then cultured in the presence of α-MEM with 15% FBS and supplemented with dexamethasone, β-glycerophosphate, and ascorbic acid. After 14–17 days, tetracycline-stained nodules were examined on the underside of the chambers using UV light.

incubation with TGF-β and more dramatically with 1 U/ml of thrombin (Figs. 9A,D). Treatment of osteogenic cells with TGF-β has been previously demonstrated to upregulate the PAR-1 transcript [3] and thus served as a useful positive control for mRNA analysis. The upregulation of PAR-1 was observed in three independent biological preparations of cells. RT-PCR analysis of primary rat stromal cells confirmed constitutive expres-

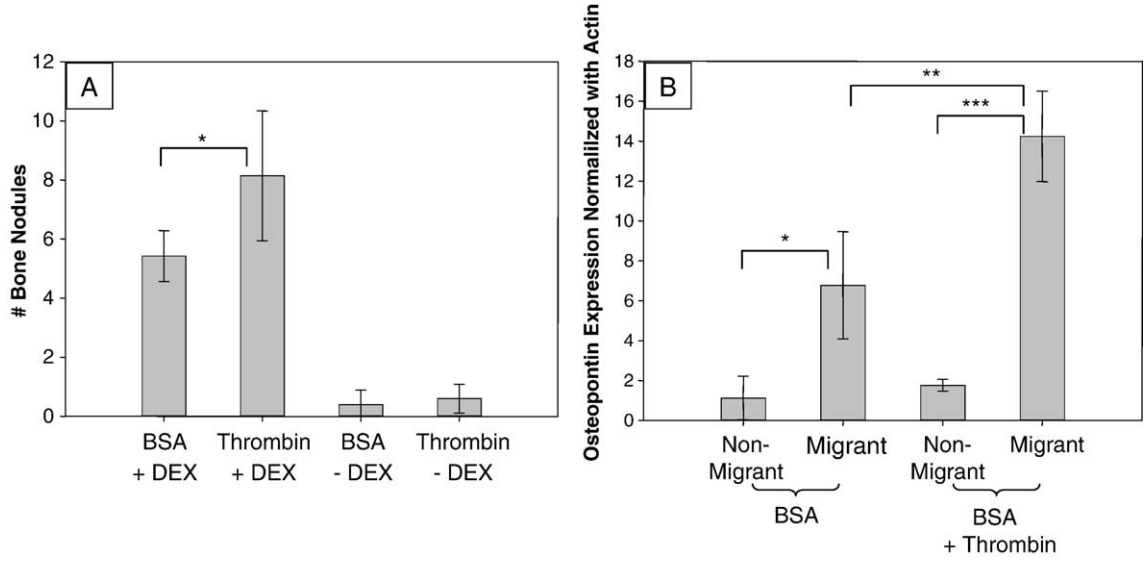

Fig. 7. Thrombin stimulates the migration of primary osteoprogenitor cells. (A) 50% more nodules formed from the migrating population, on the underside of the transwell filters, when the primary bone marrow stromal cells were subjected to a 24-h pulse of 1 U/ml thrombin compared to cells treated with a pulse of 1% BSA. A significant reduction in nodule number was observed when dexamethasone (DEX) was omitted from the osteogenic media, and a pulse of thrombin failed to stimulate more nodules for this condition. (B) The increase in nodule number observed correlated with a thrombin-induced increase in intracellular osteopontin expression in migrant cells. Specifically, thrombin stimulated over a twofold increase in osteopontin levels in migrant cells compared to cells that were treated with 1% BSA. In addition, under 1% BSA only conditions, migrating cells displayed over a fivefold increase in osteopontin levels compared to non-migrant cells whereas addition of thrombin stimulated an eightfold increase in osteopontin levels in migrant cells compared to non-migrant cells.

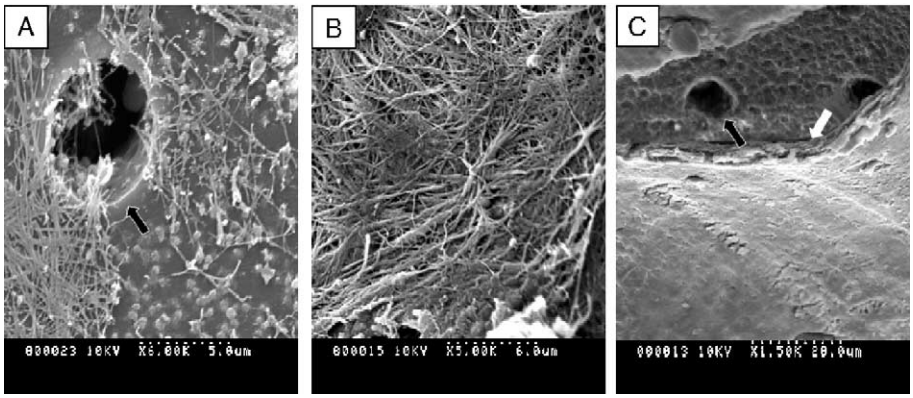

Fig. 8. Scanning electron micrographs of the matrix deposited by primary bone marrow cells culture in osteogenic media on the underside of modified Boyden chamber filters. (A) Areas containing cement line and overlying collagen were observed as well as (B) areas containing mineralized collagen. (C) A crack, most probably a result of scanning electron microscopy preparation, provides an indication of the thickness of the overlying cell layer and collagenous matrix (white arrows). Pores in the filter are illustrated with black arrows.

sion of PAR-1 mRNA, which was upregulated by thrombin treatment (Fig. 9B). Furthermore, primary rat stromal cells also constitutively expressed the transcripts for PAR-3 and MMP-2; however, neither of these genes were upregulated in response to thrombin, suggesting that they are not involved in the thrombin-mediated migration of the cells.

As in primary bone marrow stromal cells, Northern analysis of the RBMC/D8 osteogenic cell line demonstrated constitutive expression of PAR-1 and MMP-2 transcripts. Similar to the primary bone marrow stromal cells, thrombin treatment did not significantly increase MMP-2 transcripts. However, while Northern analysis demonstrated that thrombin stimulated a 50% increase in the PAR-1 transcript for the

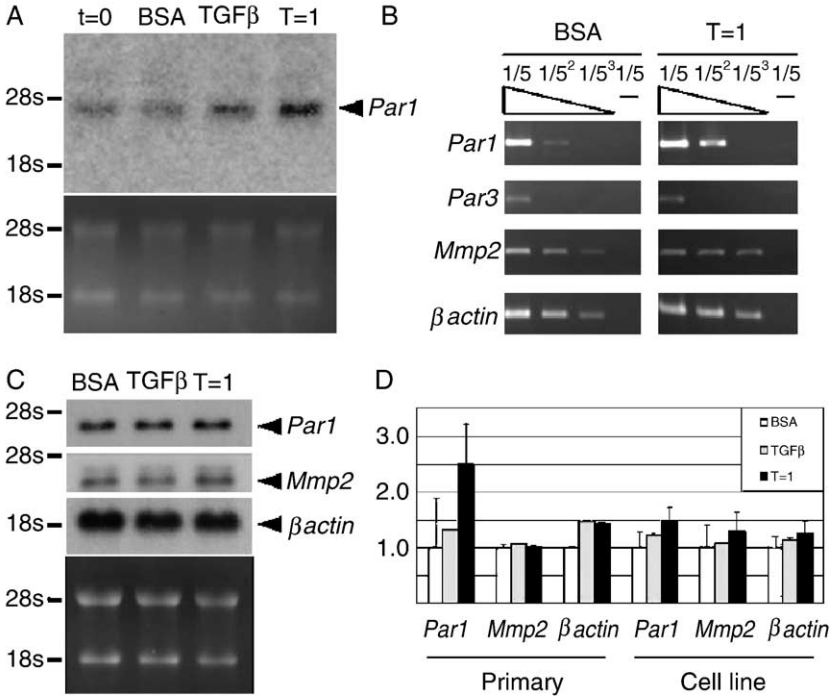

Fig. 9. Thrombin upregulates the PAR-1 transcript. Analysis of the PAR-1 transcript at $t = 0$ h and following a 24-h incubation in either: 1% BSA (BSA), 1 U/ml of thrombin ($T = 1$), or 10 ng/ml TGF-β. Northern (A) and PCR analysis (B) show that thrombin stimulates a significant increase in the expression of the PAR-1 transcript for primary bone marrow cells. Although PAR-3 is expressed by the primary bone marrow cells, its expression is not changed by the addition of thrombin in culture. (C) Northern blot analysis indicates that thrombin stimulated only a slight increase in the expression of PAR-1 for the RBMC/D8 osteogenic cell line. (D) Semi-quantitative analysis of the Northern data was used to compare the expression of PAR-1 between the two cell types. Results are shown as normalized with the 1% BSA condition, and indicate that the primary bone marrow cells were much more responsive to thrombin. Addition of TGF-β stimulated a similar response for both cells types with respect to PAR-1 and MMP-2 expression. Because thrombin and TGF-β stimulated an increase in the expression of β-actin, rRNA was used to normalize the band intensities for all conditions. Error bars represent the standard error of the mean. Results were obtained from 3 independent cell isolates for the primary cells (except for the TGF-β condition where $n = 1$) and from 2 independent cell isolates (subcultures) for the cell line.

RBMC/D8 osteogenic cell line (Figs. 9C,D), the response was not as substantial compared to the primary cells (Fig. 9D) suggesting that cell migration in response to thrombin may be mediated by the PAR-1 receptor.

Discussion

Although soluble thrombin is rapidly inactivated by endogenous plasma inhibitors such as antithrombin III [31], α-macroglobulin [31], and heparin cofactor II [41], results from numerous studies suggest that the temporal and spatial presence of thrombin is relevant to osteogenic cells during wound healing. Aside from the ubiquitous expression of thrombin at sites of vascular injury [9], and the expression of receptors for thrombin by osteogenic cells both in vitro and in vivo [2,3], thrombin may also have an indirect effect upon cell migration through the cleavage of osteopontin [42,43], which is an integral part of de novo matrix formation by osteogenic cells [16,44]. To avoid inactivation by endogenous plasma inhibitors, thrombin may bind to fibrin [9,54] (within a blood clot), while retaining its mitogenic [8] and procoagulant [9] properties. Furthermore, when ^{125}I-labeled thrombin–fibrin complexes were incubated with 10,000-fold excess of unlabeled thrombin for 3 days, less than 15% of the labeled thrombin was liberated [55]. Coincidentally, osteogenic cell invasion proceeds with granulation tissue, and granulation tissue development may be seen as early as 2 or 3 days after injury [4]. As the blood clot undergoes cell-mediated lysis, active thrombin is released into the local environment and becomes accessible to the invading cells [24].

The rapid decrease in thrombin activity as presented in Fig. 3 was likely cell mediated. This is supported by evidence that upon PAR-1 activation, thrombin [40,57] is internalized with the receptor [21], followed by thrombin degradation [40], with only a small portion of the receptors recycled to the cell surface [22]. The decrease in thrombin activity may also be due to factors released in the media such as serine protease inhibitors (i.e., protease nexin) [34].

The robust response to thrombin in the scratch wound assay by primary bone marrow stromal cells as well as the moderate response by the RBMC/D8 osteogenic cell line (Fig. 1) strongly suggests that thrombin plays a physiological role in the activation of osteoprogenitors in bony wound healing. However, because the scratch wound assay measures the degree of cell spreading which can be mediated by both proliferation and migration, we analyzed the effect of thrombin when proliferation was inhibited by mitomycin-C (Fig. 4). In fact, thrombin-induced cell spreading by primary bone marrow stromal cells was reduced by 30% by mitomycin-C, while thrombin-induced cell spreading was completely blocked in the more differentiated RBMC/D8 osteogenic cell line by mitomycin-C. This suggests that thrombin induces both proliferation and migration in primary bone marrow stromal cells; however, the action of thrombin on more differentiated osteoprogenitors may be limited to proliferation. The ability of thrombin to stimulate osteoprogenitor proliferation was confirmed by BrdU incorporation (data not shown). To confirm that the thrombin-activated migratory cells were indeed osteoprogenitors, we developed a modified Boyden chamber assay to specifically quantify migration of osteoprogenitors (Fig. 6). In fact, this novel assay demonstrates that thrombin induces migration of primary stromal osteoprogenitors (Fig. 7).

The lack of response to PPACK-treated thrombin in the absence of mitomycin-C pretreatment (Fig. 1) and in the presence of mitomycin-C pretreatment (Fig. 4) indicates that the mitogenic effects of thrombin on both cell types were dependent on thrombin proteolytic activity. Furthermore, these data also indicate that the motogenic effects on the primary cells were also mediated by thrombin proteolytic activity. This also shows that these effects were mediated via the PAR protease activated receptors, the transcripts for which are expressed by the primary cells (Fig. 9).

Thrombin stimulated the migration of the primary cells, yet had no motogenic affect on the more differentiated RBMC/D8 osteogenic cell line, thus suggesting that thrombin has a greater motogenic effect on primitive osteogenic cells (i.e., osteoprogenitors within the primary cell population) than more mature osteogenic cells (i.e., preosteoblasts representative of the cell line). The RBMC/D8 cell line represents a more mature population of osteogenic cells, compared to the majority of (primary) osteoprogenitors in the bone marrow stroma, since the cell line does not require glucocorticoids to differentiate, whereas primary marrow cells require glucocorticoids such as dexamethasone to form mineralized nodules [7].

Given the lack of specific markers for osteogenic cells, especially for more primitive osteoprogenitor cells [6], it is difficult to determine the differentiation state of osteogenic cells before the cells begin producing matrix. Therefore, the functional phenotype of the cells can only be demonstrated retrospectively through culturing the cells under osteogenic conditions and examining the surface of the culture dish for de novo bone formation [18]. By examining the number of bone nodules formed on the underside of transwell filters, an indirect measure of the number of migrating osteoprogenitors was determined. Specifically, a 24-h pulse of thrombin stimulated 50% more bone nodules which translates into a 50% increase in osteoprogenitors. Aside from developing a novel cell migration/bone nodule assay to indirectly assess the number of migrating osteoprogenitor cells, we have for the first time demonstrated that thrombin has the capacity to stimulate the migration of osteoprogenitor cells, and to increase the number of bone nodules formed by the migrated cells.

Thrombin has been demonstrated to modulate the expression of alkaline phosphatase in calvarial cells [3], suggesting a role for thrombin in mediating the differentiation of these cells into osteoblasts. However, we found

that a 24-h pulse of thrombin did not affect the number of bone nodules formed in culture (data not shown); therefore, thrombin did not exhibit any detectable effects on the differentiation of these cells. In addition, the ability of thrombin to stimulate cell proliferation did not likely influence the number of osteoprogenitor cells in these cultures since no increase in bone nodule number or size was observed. Therefore, the increase in bone nodules on the underside of transwell filters in response to thrombin (Fig. 7A) was mediated by migration of osteoprogenitors rather than proliferation or differentiation.

Interestingly, the increase in osteopontin expression observed within migrating cells (Fig. 7B) correlates with the increase in nodule number observed on the underside of filters (Fig. 7A), and with previous results for migrating stromal in general and osteogenic cells in particular [60]. Notably, thrombin is known to cleave osteopontin and to potentiate its cell binding activity through increased availability of the RGD or its cryptic sites [42,46].

In conclusion, thrombin stimulates both the proliferation and migration of primary osteogenic bone marrow stromal cells, and these effects are likely dependent on the interaction between the thrombin catalytic site and PAR-1. Thrombin was unable to illicit a motogenic effect on a more differentiated, and a more pure, population of osteogenic cells, despite having the ability to stimulate the proliferation of these cells. Our results provide evidence that thrombin can induce migration in osteogenic precursors and thus, thrombin may serve a role in stimulating the recruitment of osteoprogenitors during bony wound healing. The cell migration/bone nodule assay developed in this work offers great potential as a model system to elucidate the role of other factors that are present during the early stages of bony wound healing such as PDGF-BB, or BMP-2.

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