Platelet releasate increases the proliferation and migration of bone marrow-derived cells cultured under osteogenic conditions

Key words: bone wound healing, migration, platelet releasate

Abstract: Concentrated platelets and their products are currently being used as a clinical tool to accelerate endosseous wound healing. However, there is little understanding regarding the actions of platelets and platelet-released products on osteogenic cells. We show, herein, that releasate from thrombin-activated platelets increases the migration and proliferation of osteogenic cultures of bone marrow cells. Using a scratch wound assay, we demonstrated that platelet releasate (PR) stimulated up to a 2.4 ± 0.5-fold increase in wound closure in serum-free medium, relative to a control containing thrombin. In the presence of serum, the addition of PR resulted in a 1.45 ± 0.13-fold increase in scratch closure. To isolate cell migration from the effects of cell proliferation, cell monolayers were pre-incubated with 5, 10 and 20 μg/ml of Mitomycin C (MMC), which is a potent inhibitor of cell proliferation. This resulted in a large decrease in the leading front of scratch closure, which indicates that PR stimulated cell mitogenesis. However, irrespective of MMC pre-treatment, PR stimulated a motogenic response. These results provide evidence of possible mechanisms by which platelets could influence bone regeneration.

The recruitment and migration of osteogenic cells during fracture healing and endosseous peri-implant healing are essential for bone regeneration. It is known from in vitro experiments that these cells can be stimulated to migrate by growth factors such as transforming growth factor β, platelet-derived growth factor (PDGF), insulin growth factor, vascular endothelial growth factor, and basic fibroblast growth factor (Panagakos 1993; Lind 1998; Fiedler et al. 2002; Mayr-Wohlfart et al. 2002), all of which are released by platelets in response to injury (Pintucci et al. 2002; Weibrich et al. 2002; Martineau et al. 2004). In vivo studies have demonstrated that the application of several of these factors in combination can augment bone and soft-tissue healing (Lynch et al. 1989, 1991; Brown et al. 1994). Thus, clinicians concentrate platelets either in combination with blood plasma [platelet-rich plasma (PRP)], or as a platelet gel that is created by clotting the PRP, and apply the autologous product to sites of bone injury. In addition, concentrated platelet products can be applied as a solution containing the released growth factors [platelet releasate (PR)]. Enriched platelet preparations have been claimed to promote rapid bone healing and regeneration when added either to injury sites in isolation, or when combined with autologous bone and bone substitute materials (Marx et al. 1998; Anitua 1999; Kim et al. 2001).

Despite the great interest in using concentrated platelet preparations for clinical applications, studies in this area have been conflicting. For example, autologous grafts supplemented with PRP in mandibular...
defects in humans were reported to achieve greater trabecular bone density than the grafts alone [Marx et al. 1998], whereas combining autologous bone with PRP in cranial defects in rabbits showed no improvement [Aghaloo et al. 2002]. Additional examples of contradictory results with platelet products have been reviewed elsewhere [Sanchez et al. 2003; Freymiller & Aghaloo 2004]. Elucidating the source of these inconsistencies is difficult given the high variability in the methods used to prepare the platelet products, which is complicated further by the lack of understanding of the mechanisms by which platelets mediate cell activity (Schmitz & Hollinger 2001; Sanchez et al. 2003).

Recently, several in vitro studies have been performed to elucidate the effects of platelets and their products on osteogenic cells [Kawase et al. 2003; Arpornmaeklong et al. 2004]. Although various platelet preparations have been shown to stimulate the in vitro proliferation of bone marrow and periosteal cells [Gruber et al. 2002, 2003; Lucarelli et al. 2003; Arpornmaeklong et al. 2004], no studies have suitably isolated the effects of platelets or their products on osteogenic cell migration, which is critical for the osteoconduction phase of endosseous wound healing [Davies 2003]. Thus, the aim of the current investigation was to determine the effect of PR on the migration of bone marrow cells [BMCs] cultured in osteogenic conditions. We hypothesized that, in addition to the ability of PR to stimulate cell proliferation, PR could stimulate cell migration. Specifically, we wished to demonstrate that migratory stimulation in the presence of PR would be greater than that observed in the presence of thrombin, which is a component of PR that is known to increase cell motility [Karp et al. 2004].

Material and methods

Materials

For this study, the monoclonal antibody GPIIb/IIIa, directly conjugated to fluorescein isothiocyanate (FITC), was purchased from Southern Biotechnology Associates [Birmingham, AL, USA], while the immunoglobulin G (IgG) isotype control was obtained from BD Biosciences [Mississauga, ON, Canada]. The calibrated size beads used to set the flow cytometry gates were obtained from Polysciences [Warrington, PA, USA]. Bovine serum albumin (BSA) was purchased from Sigma Chemical Company [St Louis, MO, USA]. The MTS solution [CellTiter 96 "Aqueous Non- Radioactive Cell Proliferation Assay], which consists of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)3H-tetrazolium, inner salts MTS and the electron coupling reagent phenazine methosulphate (PMS), was purchased from Promega Corporation [Madison, WI, USA]. A VERSAmax tunable microplate reader [Molecular Devices Corporation, Sunnyvale, CA, USA] was used to obtain the absorbance readings.

Cell isolation and culture

Rat bones were excised from young Wistar rats [115–130 g] [Charles River, St Constant, QC, Canada] and transferred to 1 ml of an antibiotic/fungizone solution consisting of penicillin G [1670 U/ml], gentamicin [500 µg/ml] and amphotericin B [1 µg/ml] (Sigma). After washing three times, epithyes were removed, and the marrow from each diaphysis was flushed with culture medium. The latter consisted of a minimal essential medium [Faculty of Medicine, University of Toronto, Toronto, ON, Canada] supplemented with 15% [vol/vol] fetal calf serum [StemCell Technologies Inc., Vancouver, BC, Canada], 10^{-8} M dexamethasone (Sigma) and 10% [vol/vol] of the same antibiotic solution used for washing. Marrow cells of both diaphyses were collected and cultured in the same medium in Falcon T75 flasks [Becton Dickinson Labware, Franklin Lakes, NJ, USA]. Cultures were maintained at 37°C in an incubator with an atmosphere consisting of 95% air, 5% CO2 and 100% relative humidity. The culture medium was changed every 2 or 3 days. On day 5 or 6 after isolation, upon reaching ~80% confluence, the cells were subcultured by enzymatic release using 0.01% trypsin [GIBCO BRL now Invitrogen Life Technologies, Gaithersburg, MA, USA] for 12–15 min.

PR

Whole blood was obtained from consenting, healthy volunteers and mixed with acid citrate dextrose (ACD-A) anticoagulant [Sigma] [1:10 vol/vol]. The blood was centrifuged at 200g for 10 min to pellet the red and white blood cells, and to enrich the plasma with suspended platelets [PRP] to which 0.2 µg/ml prostaglandin E1 [PGE1] (Sigma), which is an inhibitor of platelet aggregation, and ACD-A [1:10 vol/vol] were added. This was further centrifuged at 900g for 5 min to pellet the platelets. The pellet was resuspended in HEPES Tyrode’s buffer (HTB) [137 mM NaCl, 2.7 mM KCl, 16 mM NaHCO3, 5 mM MgCl2, 3.5 mM HEPES, 2.5 mM CaCl2, 5.5 mM Glucose, 2 mg/ml BSA, pH 7.4] to obtain a final concentration of 1000 × 10^5 platelets/ml. This represented a 49% increase over the average physiological concentration of collected platelets [214 ± 37 × 10^5 platelets/ml]. The platelets were activated with 1 National Institute of Health [NIH] U/ml of bovine thrombin (Sigma) for 30 min at 37°C. The supernatant containing the released platelet factors was removed from the platelet clumps and used immediately, or frozen at −70°C for up to 3 months. For proliferation and scratch wound assays, PR was diluted with either serum-free medium [SFM] containing 0.75% BSA or serum-containing medium [SM], to obtain released factors/ml from: 500 × 10^6 platelets [1:2], 250 × 10^6 platelets [1:4], 25 × 10^6 platelets [1:40], 5 × 10^6 platelets [1:200], 2.5 × 10^6 platelets [1:400] and 0.25 × 10^6 platelets [1:4000]. The corresponding buffer controls used in the scratch wound and proliferation assays consisted of HTB-containing thrombin diluted in medium at concentrations equivalent to those of the PR. For several of the conditions examined, SM and SFM alone were used as additional controls.

Flow cytometry

Platelet activation was confirmed by flow cytometry [Coulter² EPICS XL³, Coulter Corporation, Miami, FL, USA]. Five microliters of the resting or activated platelet suspension [1000 × 10^6 platelets/ml] was added to 50 µl HTB containing a saturating concentration of FITC-labelled anti-GPIIb/IIIa antibody. After a 20 min incubation at room temperature, the samples were fixed with 1% paraformaldehyde and analyzed. Data for FITC fluorescence and forward scatter were obtained in the logarithmic mode. FITC-positive platelet-specific events were corrected for non-specific binding with a fluorescently labelled IgG.
isotype control. The vertical gate separating the platelet microparticles [PMPs] and platelets was set with calibrated size beads [0.2–0.8 μm], thereby distinguishing the two populations on the basis of size. Five thousand positive platelet-specific events were analyzed, and the PMPs were expressed as a percentage of the total platelet-specific events.

Cell proliferation
The cells were plated at a density of 10,000 cells/cm² in 96-well plates in culture medium. After an overnight incubation, the medium was changed to SFM. Cells were then incubated for 18 h in the presence of PR diluted in SFM. To determine the proliferative effect of PR, an MTS solution diluted in medium was added to the cells for 3 h at 37°C. An ELISA microplate reader was used to measure the absorbance of the soluble formazan product at 490 nm.

Scratch wound assay
Rat BMCs were seeded at 6000–10,000 cells/cm² in 96-well plates in culture medium. After allowing the cells to attach for 24 h, the medium was removed, and the cells were incubated with MMC under the same conditions as described above. The control consisted of cells that were not pre-incubated with MMC. The cells were allowed to grow for 6 days, at which point they were fixed with 3.7% formaldehyde for 10 min. After washing twice with PBS, the cells were stained with toluidine blue and imaged by light microscopy.

Statistical analysis
For the proliferation and scratch wound assays, measurements were collected from at least three experiments, with three to six replications within each experiment. General linear modelling with analysis of variance (ANOVA) was applied for the dependent variable in each case, which was either cell proliferation, leading front of scratch closure or fold-increase in scratch closure, with the respective doses of PR as the independent factors (SAS, version 8, Cary, NC, USA). Overall differences were determined using the least-square means estimates from the various models. A multivariate repeated measures ANOVA was used for the analysis of the effect of PR in the scratch wound assay over time. Student’s t-test was used to make single pairwise comparisons. The results are expressed as means ± standard deviations in the text, and as means ± standard errors of the means in the graphs. A P-value of less than 0.05 was considered as significant.

Results
Stimulatory effect of PR in SFM
Flow cytometric analysis confirmed that 1 U/ml of thrombin efficiently activated the platelets and generated PMPs [Fig. 1a and b], which were defined to be less than 0.8 μm in size in accordance with previous reports [Hagerstrand et al. 1996]. Less than 6% PMPs were identified in the resting platelet population, whereas PR contained more than 96% PMPs.

The addition of different dilutions of PR to SFM resulted in a linear dose–response increase in BMC proliferation [Fig. 2] (P = 0.0002), which was significantly greater than SFM with thrombin-containing buffer (P = 0.0001). The maximum response was observed at a PR dilution of 1:2, where proliferation was increased by 2.15 ± 0.6-fold in relation to the thrombin-buffer control [Fig. 2].

A striking difference in scratch closure was seen after 18 h between the PR and the thrombin-buffer control group at a dilution of 1:4 [Fig. 3a]. Quantitative analysis demonstrated that PR stimulated scratch closure over time [P = 0.06], which trans-
lated into a 106.94 ± 55.27% and a 140.61 ± 55.84% increase in scratch closure at 12 and 18 h, respectively (Fig. 3b). Furthermore, there was a significant linear effect of time ($P = 0.0049$). Unlike the thrombin-buffer control group, the rate of scratch closure (slope of the leading front of scratch closure vs. time) in the PR-treated group continued to increase up to 12 h.

To determine the effect of thrombin-containing controls on scratch closure in SFM, the results were normalized either with medium alone, or with medium in the presence of thrombin and buffer. The addition of PR to SFM resulted in a linear ($P = 0.002$) and quadratic ($P = 0.01$) dose-dependent increase in scratch closure (Fig. 4). PR-stimulated scratch closure relative to both SFM alone and SFM containing thrombin buffer ($P < 0.0001$ for all conditions). In addition, the response normalized with respect to SFM was significantly greater than the response standardized with SFM in the presence of thrombin buffer ($P = 0.0001$). At a PR dilution of 1:2, scratch closure was increased by 4.7 ± 1.39-fold in SFM and by 2.04 ± 0.28-fold in SFM with the thrombin-buffer control. The incorporation of thrombin-containing controls thus resulted in a 128.75 ± 41.65% and a 93.85 ± 35.87% reduction in response at a 1:2 and a 1:4 PR dilution, respectively. Interestingly, no significant difference was found between groups treated with PR at dilutions of 1:2, 1:4 and 1:40, compared with SM with 15% serum, which stimulated a 3.41 ± 0.76-fold increase in scratch closure ($P > 0.05$ vs. PR groups, data not shown).

**Fig. 2.** Cell proliferation as a function of platelet releasate (PR) dilution. Bone marrow-derived cells were incubated in the presence of PR, or in the presence of thrombin-buffer controls for 18 h. The results are expressed as fold increases in proliferation over the thrombin-buffer controls. The data represent means ± standard error of means ($n = 4–6$).

**Fig. 3.** Scratch closure at: (a) 0 and 18 h in response to a physiological concentration of platelet releasate (PR) and to the thrombin-buffer control [1:4 dilution]. The edges of the scratches at time 0 have been marked with dotted lines for clear visualization of the infiltration of cells into the denuded area as a result of migration and proliferation. The scale bar in each image represents 245 μm. (b) There is a significant difference between the leading front of scratch closure at 6, 12 and 18 h between the PR-treated groups (○) and the thrombin-buffer control groups (■). Each point represents the mean ± SEM of four experiments ($n = 4$) each comprising triplicate scratches in two wells of a six-well plate (total = 6).

**Stimulatory effect of PR in SM**

In order to use more physiologically relevant conditions, the scratch wound assay was also performed in the presence of serum. PR at a dilution of 1:40 stimulated scratch closure in SM ($P < 0.0001$) (Fig. 5). The most noticeable effects were observed in the presence of 2%, and 5% serum, where scratch closure increased by 32 ± 5% ($P = 0.003$) and 27 ± 11% ($P = 0.032$), respectively. However, in the presence of 15% serum, scratch closure was not significantly influenced by PR. The addition of thrombin buffer to SM did not change the cell response ($P = 0.37$).

The effect of different dosages of PR on scratch closure was subsequently assayed
in the presence of medium containing 2% serum. The addition of PR to this medium resulted in a significant linear dose–response increase in the response ($P = 0.0045$). PR at a dilution of 1:2 stimulated a maximal increase in scratch closure of $1.45 \pm 0.13$-fold when normalized with 2% serum alone, and with 2% serum with thrombin buffer, respectively (Fig. 6). While both of the latter two conditions were significantly greater than the controls ($P < 0.0001$), there was no variation between them ($P = 0.54$).

### Relationship between migration and proliferation

When the cells were examined 6 days after treatment with 5, 10 or 20 μg/ml MMC, we observed that cell confluence in all of the treated wells was greatly reduced compared with the untreated wells. Furthermore, we observed that as the MMC concentration increased, cell density decreased (Fig. 7). The addition of PR to SFM or SM at a 1:40 dilution stimulated significant increases in scratch closure at all MMC pre-incubation concentrations, compared with the respective SFM and SM controls ($P = 0.0001$ for both conditions) (Fig. 8). This ranged from $238 \pm 58\%$ (0 μg/ml MMC) ($P = 0.001$) to $140 \pm 76\%$ (20 μg/ml MMC) ($P = 0.004$) in SFM and from $35 \pm 17\%$ (0 μg/ml MMC) ($P = 0.013$) to $32 \pm 14\%$ (20 μg/ml MMC) ($P = 0.037$) in SM.

A marked decrease in the leading front of scratch closure was also observed after MMC pre-treatment (Fig. 8). In SFM, in the presence of PR, the leading front decreased from $269 \pm 96\mu$m, in the group with no MMC, to $51 \pm 3\mu$m in the condition that was treated with 20 μg/ml MMC (Fig. 8a). In SM, the leading front decreased from $409 \pm 39$ to $245 \pm 41\mu$m between the same groups (Fig. 8b). When PR at dilutions ranging from 1:4000 to 1:2 were added to cells pre-incubated with 20 μg/ml MMC, a similar dose response as obtained in Fig. 4 was observed (data not shown).

### Discussion

We have shown that PR stimulates both the proliferation and migration of rat bone marrow-derived cells in the presence of dexamethasone, which is known to upregulate osteogenic cell differentiation (Herbertson & Aubin 1995; Ohgushi et al. 1996). Although PR has recently been demonstrated to increase rat bone marrow-derived cell invasion into fibrin gels (Oprea et al. 2003), the effects of PR on cell migration were not distinguished from proliferation. It therefore cannot be determined, as to which of these two responses contributed to the increased cell numbers observed within the gel. Furthermore, PR has been shown to stimulate human BMCs to traverse through 8 μm pore-size filters within a modified Boyden Chamber assay (Gruber et al. 2004). However, the freshly trypsinized cells were not allowed to attach to the filters prior to subjecting the cells to PR. It is thus difficult to establish whether the increased response was because of the influence of PR on cell adhesion or migration. To our knowledge, the present study is the first to isolate the effects of PR on cell migration from those of cell proliferation within osteogenic cultures. This was achieved through the inhibition of cell proliferation with MMC. In addition, we accounted for adhesive effects by allowing the cells to grow until confluence, prior to examining cell migration.

PR stimulated scratch closure up to 2.4-fold when compared with the thrombin-buffer controls in SFM; however, in SM, scratch closure was only increased up to 1.4-fold. Antibodies against PDGF have been demonstrated to diminish the migratory response of vascular smooth muscle cells to serum, indicating that PDGF within serum promotes the motility of these cells (Bernstein et al. 1982). Thus, in the presence of serum, the response was greater than in SFM as it was already stimulated because of the presence of PDGF and other growth factors. With the addition of PR, it is likely that the response became saturated, and while PR stimulated a response in SM, the response was not as great as that observed in SFM. The presence of dexamethasone in the cultures ensured a more differentiated cell population. However, in less differentiated conditions, it could be expected that PR would stimulate an even greater migratory response (Fiedler et al. 2002).

Thrombin is typically used to generate PR and to form platelet gels. However, no studies involving platelet products have used controls for thrombin. In the current study, we distinguished the specific effects of PR by adding the respective concentrations of thrombin, contained within the various dilutions of PR, to the thrombin-buffer controls. The results demonstrated that the response normalized with SFM was significantly greater than the response.
standardized with the thrombin-buffer controls. This suggests that thrombin increased scratch closure in SFM. However, in SM, there were no differences between the responses normalized with either thrombin-containing controls or with medium alone. This indicates that, at the investigated concentrations of PR, thrombin had no effect in the presence of serum. This may be attributed to antithrombin III and α2-macroglobulin contained within serum (Ganrot & Schersten 1967; Seegers 1975), which can bind to thrombin and inhibit its actions. Previous reports have determined that PR increases mitogenic and motogenic cell activity in both SFM and SM (Gruber et al. 2002; Oprea et al. 2003; Arpornmaeklong et al. 2004). Based on the current findings, it is conceivable that in SFM, PR would have stimulated a lower response if controls for thrombin were used in these studies; yet, in SM it is unlikely that thrombin within PR significantly influenced the results.

A limitation of using controls containing thrombin was that the final concentration of thrombin in PR was likely higher than that within the thrombin-buffer controls. During platelet activation, thrombin interacts with receptors on the platelet surface, which may reduce the soluble content in PR (Yamamoto et al. 1991; Brass 2003). Thus, it is possible that the thrombin-buffer controls overcompensated for the effects of thrombin in the PR. Nevertheless, in all cases, PR stimulated cell responses that were greater than those observed with the thrombin-buffer controls. Furthermore, our controls did not account for the small amount of PGE1 remaining in the supernatant following centrifugation, although Bray & Franco (1978) reported that PGE1 had no significant inhibitory or chemotactic effects on cell migration.

To establish that the enhanced rate of scratch closure was not only a result of increased cell proliferation, we pre-incubated with MMC. It is known that the concentration of MMC required to inhibit DNA synthesis varies depending on the cell type (Wu et al. 1999; Ponchio et al. 2000). As BMCs consist of a heterogeneous population, MMC likely exerted differential effects on inhibiting cell mitogenesis. Thus, cells were pre-incubated with 5, 10 or 20 μg/ml MMC to reduce proliferation incrementally. By pre-treating the cells with MMC, we were able to establish that scratch closure was because of both cell proliferation and migration. However, given the large decrease in the leading front observed with increasing MMC concentration, the proliferation response likely contributed more to scratch closure. Nevertheless, as 5–20 μg/ml MMC prevents cell confluence, and since cell mitogenesis is typically inhibited with these concentrations (Pepper et al. 1989; Horodyski & Powell 1996; Kapur et al. 2002), it is likely that at 20 μg/ml, most of the cells were not dividing. Thus, the increase in response to PR with 20 μg/ml MMC pre-treatment was presumably due mainly to the influence of PR on cell migration. This indicates that, in addition to the proliferation response, PR stimulated migratory activity.

In summary, we have demonstrated that PR increases both the migration and proliferation of BMCs cultured in osteogenic conditions in both SM and SFM. Through inhibition of proliferation with MMC, we isolated the effects of PR on cell migration. Furthermore, these results demonstrate that PR stimulates a greater cell response than thrombin alone.

Acknowledgements: This study was supported by an Ontario Research and Development Challenge Fund (ORDCF) grant and an unrestricted education grant from Implant Innovations Inc., FL, awarded to JED.
References


