Chemotactic and Mitogenic Stimulation of Human Mesenchymal Stem Cells by Platelet Rich Plasma Suggests a Mechanism for Enhancement of Bone Repair

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INTRODUCTION

Platelets are known to perform multiple functions during injury and tissue repair. While their role in hemostasis is well understood, their mechanism of action in promoting wound healing requires further characterization. As a repository of multiple growth factors such as PDGF, EGF, VEGF, and TGF-ß, degranulation of platelets at wound sites serves to initiate or enhance the healing cascade. Armed with this knowledge, clinicians have used platelet concentrates in conjunction with bone graft materials to enhance osseous repair[1,2]. In addition, experimental evidence has shown that when PDGF or platelet concentrates are combined with demineralized bone or certain other materials, augmentation of bone formation ensues[3].

The purpose of this study was to begin elucidating the cellular mechanisms that underlie these observations. Since mesenchymal stem cells (MSCs) are known to be an essential component of the tissue repair process, we sought to characterize elements of their response to platelet concentrates in the controlled in vitro environment.

Donor Preps (n)	Donor Age (years)	Hematocrit (%)	Initial Platelet Count ($x10^3/\mu l$)	Platelet Yield (%)
21	30 ± 6.2	38.6 ± 2.9	223.3 ± 45.80	70.6 ±11.0

Isolation of Concentrated Platelets using the SYMPHONY Platelet Concentration System. Values equal averages ± S.D.

PLATELET CONCENTRATION

Platelet rich plasma (PRP) was isolated from approximately 55 ml of fresh human blood (IRB-approved protocol) using the Symphony™ Platelet Concentration System (DePuy AcroMed, Raynham, MA), designed to be used at the point-of-care for obtaining a platelet concentrate from a small amount of blood. Samples of the starting material and platelet concentrates were analyzed to determine the absolute concentrations and yields of platelets. PRP, platelet poor plasma (PPP) and whole blood were clotted with thrombin (1000 U/ml in 10% $CaCl_2$) by adding 1 part thrombin stock solution to nine parts PRP, PPP or blood to yield a final thrombin concentration of 100 U/ml. The soluble platelet releasates from the clotted preparations were isolated by centrifugation and cleared by ultrafiltration.

PRP and PPP releasates were diluted in serum-free DMEM to generate appropriate final dilutions of platelet releasate. Similar to previously published results[4], we obtained very high efficiency of platelet concentration. Also, the efficiency of the platelet concentration was reproducible across the various samples as can be seen by the low standard deviation. The specific levels of various growth factors were not measured in this study. However, it has been previously reported that in platelet concentrates processed using the current system, the concentration of growth factors increase linearly with the platelet concentration[4,5].

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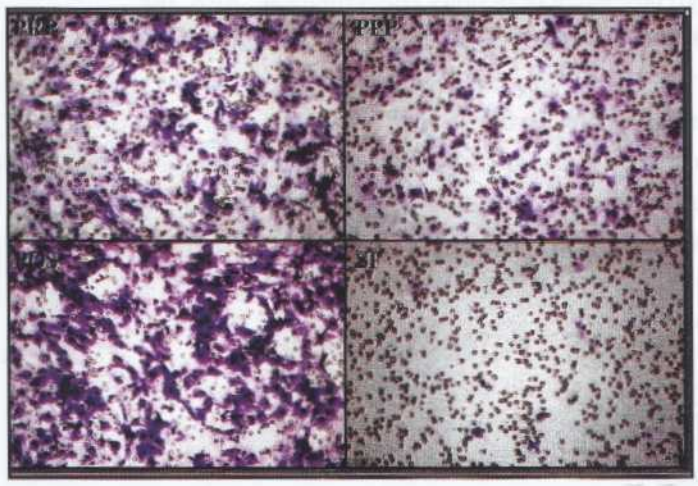
CELL PREPARATION

In order to evaluate the mitogenic activity of PRP, human MSCs (hMSCs) were isolated and culture-expanded from bone marrow (IRB-approved protocol) using published techniques[6]. The growth media (GM) for the selection and culture-expansion of hMSCs consisted of DMEM supplemented with 10% fetal bovine serum (FBS). The specific lot of FBS was chosen for it's ability to optimize MSC selection and growth[7].

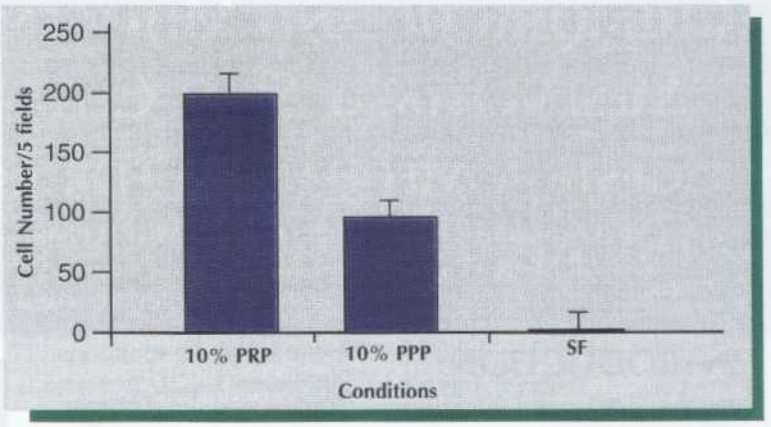
Control media consisted of serum free medium (SF), or DMEM supplemented to 10% (v/v) with the following preparations: PPP releasate alone, or serum from clotted peripheral blood (PB). Test media consisted of DMEM supplemented with undiluted PRP releasate or PRP releasate diluted with PPP, such that the final concentration of PRP releasate ranged from 0.625- to 10-fold of that in media supplemented with peripheral blood. To achieve the 5-fold concentration and the 10-fold platelet concentration, the PRP releasate was added to the media at 10% and 20% (v/v), respectively. To achieve the lower platelet concentrations, PRP diluted with an appropriate amount of PPP was added to the media at 10% (v/v).

CHEMOTACTIC MIGRATION

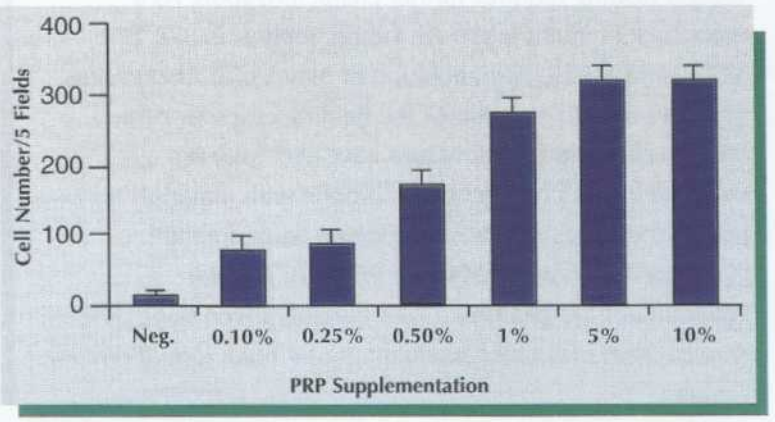
The ability of concentrated platelet releasate to stimulate the chemotactic migration of hMSCs was measured using a Neuroprobe AC48 Boyden Chamber with 5 µm pore size polycarbonate filters. 7,500 hMSCs in 50 µl serum-free medium were added to the upper chambers of each well. Lower chambers contained test media. Cells were allowed to migrate for 4 hours at 37° C, at which time non-migratory cells were scraped from the filter. Migratory cells on the underside were stained with crystal violet dye and counted. PRP releasate and VEGF each stimulate chemotactic migration of hMSCs in a dose-dependent manner. Since VEGF is a component of PRP releasate, it is at least partially responsible for the chemotactic activity of PRP releasate on hMSCs.



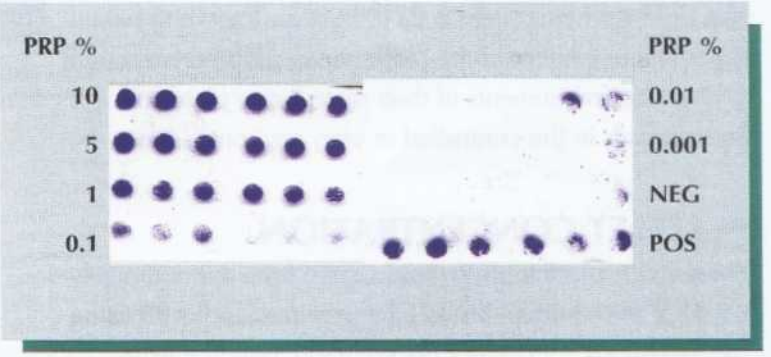
Photomicrographs of hMSCs after chemotaxis due to PRP releasates and proper controls(original magnification 200x).



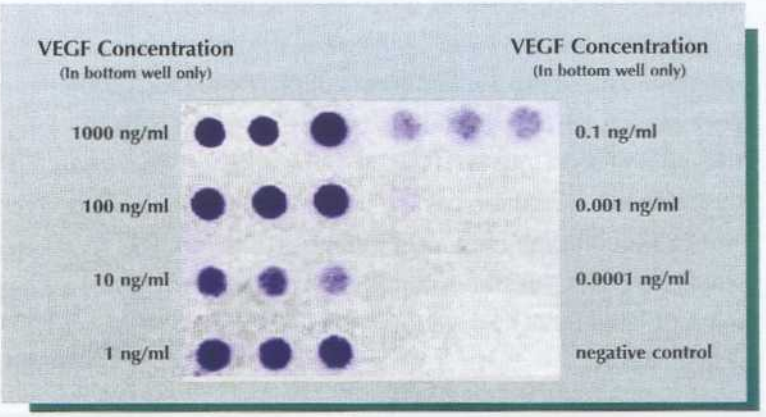
Chemotactic Migration of hMSCs in Response to Various Media Additives



Chemotactic Migration of hMSCs in Response to PRP Releasate



PRP-Releasate Stimulates Dose-Dependent Chemotactic Migration of hMSCs



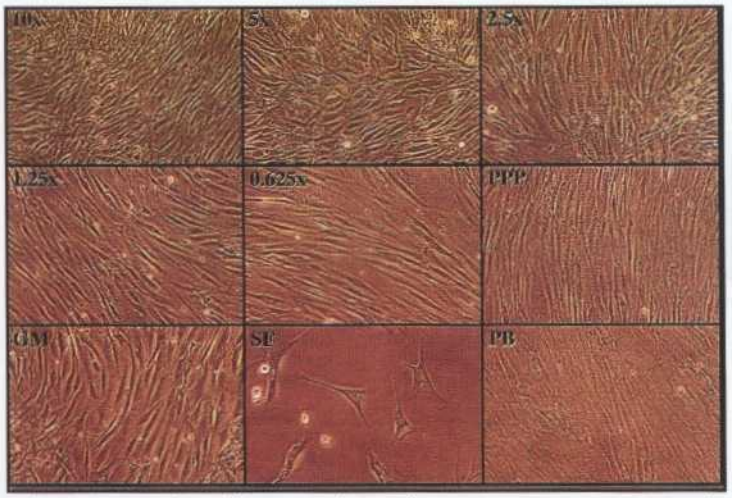
VEGF Stimulates Dose-Dependent Chemotactic Migration of hMSCs

However, extrapolating from the reported levels of VEGF in the PRP[4], it is clear that VEGF by itself is unlikely to account for the majority of the chemotactic effects of PRP. Other known chemotactic molecules, such as TGF-β probably contribute to the chemotactic response of PRP.

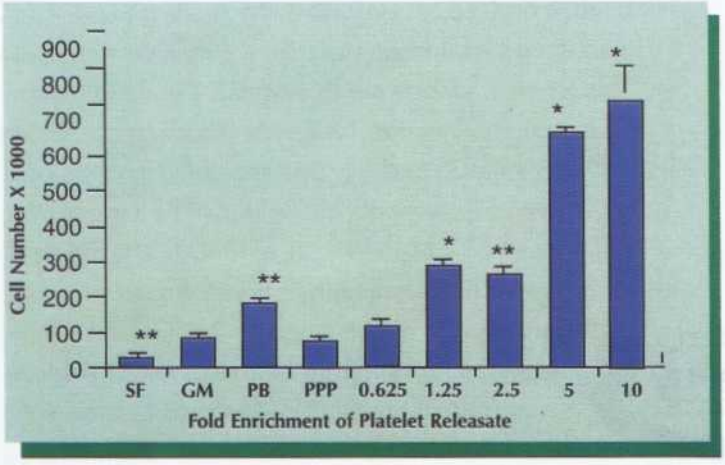
MITOGENIC STIMULATION

In order to evaluate the mitogenic activity of PRP, second passage hMSCs were replated at a density of $3x10^3/cm^2$ in serum-free DMEM. Cells were allowed to attach and incubate for 48 hours, at which time culture medium was replaced with the various media. hMSCs were allowed to incubate in test and control media for 7 days with complete media changes taking place on day 4. At the end of the 7 day time course, cells were released with trypsin and counted with a hemocytometer.

PRP releasate stimulates proliferation of hMSCs in a dose-dependent manner. While these experiments demonstrate that serum from a fresh human blood clot, and even PPP, can stimulate hMSC proliferation, approximately 90% of the mitogenic activity in PRP is derived from the platelet releasate.



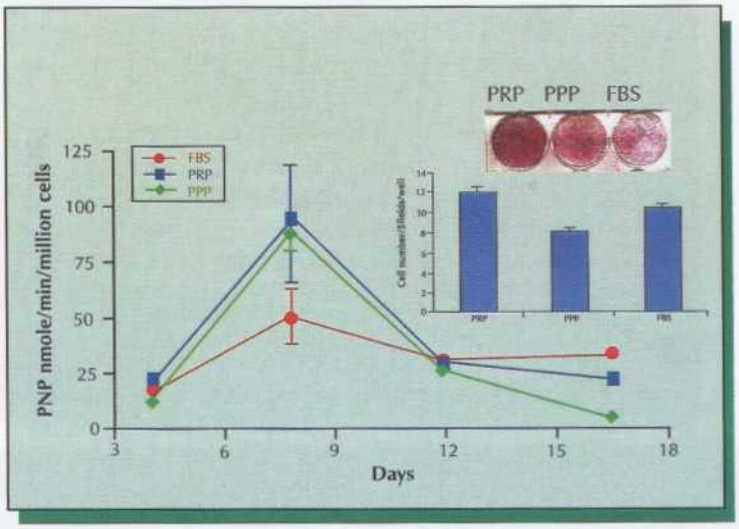
Photomicrographs of hMSCs cultivated in various concentrations of platelet releasate or appropriate controls (original magnification 200x).



*Dose-Dependent Mitogenic Effects of Platelet Releasate on hMSC's. Statistical differences (two-tailed paired t-tests) are shown relative to hMSC Growth Medium (GM) control, *p< 0.05 and **p<0.01*

OSTEOGENIC DIFFERENTIATION

The ability of PRP to support mitotic expansion of hMSCs without loss of their osteogenic potential was demonstrated by first expanding hMSCs in DMEM-LG supplemented to 10% with 5x PRP releasate, PPP releasate, or GM. After 5-7 days of mitotic expansion in the various test media, hMSCs were harvested and reformatted at $3x10^3/cm^2$ and allowed to attach overnight in serum-free DMEM-LG. The next day, culture media were switched to standard GM plus Osteogenic Supplements (OS) consisting of 10^{-7}M dexamethasone, 10m M beta-glycerophosphate and 50 µM ascorbic acid-2-phosphate. On days 4, 8, 12 and 16 cultures were analyzed for alkaline phosphatase expression and calcium deposition into the cell layer.



Osteogenic Differentiation of hMSCs after PRP Releasate-induced Mitotic Expansion

As expected, PRP releasate by itself does not cause osteogenic differentiation of hMSCs. In hMSC samples grown in the presence of PRP releasate without OS, the dominant effect was proliferation with no evidence of differentiation. This trend continued in samples grown with PRP (or PPP) releasates plus OS, as cell proliferation was nearly double that observed in GM plus OS samples. The net effect of this potent mitogenic activity was to keep cells cycling, thus preventing their entry into the osteogenic differentiation pathway.

In samples that were exposed to osteogenic differentiation signals, after rapid expansion in the PRP-supplemented media, the levels of various osteogenic markers were similar or greater than those observed in the controls when normalized to a per cell level. Thus, mitogenic stimulation of hMSCs by PRP releasate occurs without alteration of the cell's phenotype or the loss of its osteogenic development potential. Furthermore, the proliferation rate continued to be higher in the samples initially expanded in PRP, thus leading to an overall increase in osteogenic matrix output in these samples as compared to the controls. This effect was similar to data generated by Slater, et al. using human fetal osteoblastic cells.

CONCLUSIONS & DISCUSSION

- PRP releasate and Vascular Endothelial Cell-Derived Growth Factor (VEGF) each stimulate chemotactic migration of hMSCs in a dose-dependent manner.
- PRP releasate stimulates proliferation of hMSCs in a dose-dependent manner. Approximately 90% of the mitogenic activity in PRP is derived from the platelet releasate.
- Mitogenic stimulation of hMSCs by PRP releasate occurs without alteration of the cell's phenotype or loss of its osteogenic developmental potential.

These observations are consistent with in vivo wound healing models in which degranulated platelets initiate or enhance the healing cascade through the transient chemotactic attraction and mitotic stimulation of reparative cells, which is then followed by morphogenic signals from other sources that induce cell differentiation.
These studies represent the first published data showing a direct effect of PRP releasate on purified human MSCs, which play a pivotal role in the process of musculoskeletal tissue repair. The observation that this easily prepared, autologous source of concentrated growth factors possesses chemotactic and mitogenic activity lends further credence to its therapeutic role in clinical orthopaedics. In view of the data presented, we suggest that local application of PRP causes migration of hMSCs to the wound site, followed by their massive replication to form a repair blastema. As the bioactive factors diffuse away from the fibrin scaffold, now densely populated by hMSCs, the cells cease dividing and are primed to respond to the endogenous inductive cues that stimulate differentiation. The local and transient activity of PRP in this model of tissue repair is responsible for initiating and accelerating the natural healing cascade.

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