

Platelet Microparticles Promote Neural Stem Cell Proliferation, Survival and Differentiation

Yael Hayon · Olga Dashevsky · Ela Shai · David Varon · Ronen R. Leker

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Abstract Platelet microparticles (PMP) are small subcellular fragments, shed upon platelet activation. PMP host a variety of cytokines and growth factor that were previously shown to affect angiogenesis and postischemic tissue regeneration. This study attempted to explore the effect of PMP on neural stem cell (NSC) proliferation, survival and differentiation. Cells were grown as neurospheres and treated with PMP, or relevant growth factors, sphere size and cell fates were evaluated. PMP treatment led to larger neurospheres with increased cell survival. PMP treatment was comparable with the effect of acceptable single growth factors such as fibroblastic growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). PMP treatment also increased the differentiation potential of NSC to glia and neurons. Specific growth factor inhibitors only partly blocked these effects, which were associated with increments in ERK and Akt phosphorylation. In this study, we show that various

growth factors contained within the PMP promote neuronal cell proliferation, survival and differentiation. The results suggest a role for platelet microparticles in augmenting endogenous neural progenitor and stem cells angiogenesis and neurogenesis that might be utilized for treatment following brain injury.

Keywords Angiogenesis · Neurogenesis · Platelets · Microparticles · Cerebral ischemia

Introduction

Platelets are essential players in primary hemostasis, initiating clot formation in injured vessels. During the last decade, platelets were also shown to have a role in inflammation responses, angiogenesis, wound healing, and tissue regeneration due to their proximity and interaction with the endothelium and their abundant growth factors and chemokines (Gimbrone et al. 1969; Banks et al. 1998; Peterson et al. 2010). Upon activation, platelets shed microvesicles (0.1–1 μm in size) and exosomes (~ 40–100 nm), usually referred to as platelet-derived microparticles (PMP) (Heijnen et al. 1999). The role of PMP in disease development is unknown, but the composition of PMP in the plasma of patients varies considerably depending on the severity of the pathology (Helley et al. 2009).

In the brain, it was shown that PMP can be internalized in vitro by human brain endothelial cells (EC) in a process of active endocytosis (Faille et al. 2011) and may contribute to the pathogenesis of cerebral malaria by increasing the adherence of infected red blood cells to EC (Faille et al. 2009). Microparticles were also suggested to be involved in microvascular thrombosis contributing to ischemic brain disease

Y. Hayon · R. R. Leker
Department of Neurology, Peritz and Chantal Scheinberg
Cerebrovascular Research Laboratory,
Hadassah Hebrew University Medical Center,
Jerusalem, Israel

O. Dashevsky · E. Shai · D. Varon
Department of Hematology, Coagulation Unit,
Hadassah Hebrew University Medical Center,
Jerusalem, Israel

R. R. Leker (✉)
Stroke Service and the Peritz and Chantal Cerebrovascular
Research Laboratory,
Hadassah Ein Kerem, P. O. Box 12000, Jerusalem 91120, Israel
e-mail: leker@hadassah.org.il

D. Varon (✉)
The Lester Stromer Coagulation Unit,
Hadassah Ein Kerem, P. O. Box 12000, Jerusalem 91120, Israel
e-mail: dvaron@hadassah.org.il

as well as to neuroinflammatory conditions (Horstman et al. 2009).

On the other hand, we and others have shown that PMP, in addition to their procoagulant and inflammatory effects, promote all stages of angiogenesis *in vitro* and *in vivo* (Baj-Krzyworzeka et al. 2002; Kim et al. 2004; Brill et al. 2005; Mause et al. 2010).

The role of PMP in blood vessel development was first shown by Kim et al. (2004). They showed involvement of PMP in EC proliferation and tubule formation, as well as anti-apoptotic and chemotactic effects of PMP on EC. These effects were mediated by growth factors and lipid components contained in the PMP (Janowska-Wieczorek et al. 2005; Mause et al. 2010).

PMP not only passively carry various proteins and receptors but also chemoattract hematopoietic cells and stimulate their adhesion, survival, engraftment, and proliferation (Baj-Krzyworzeka et al. 2002).

Platelet-derived microparticles were shown to modulate functional features of endothelial progenitor cells crucial for their regenerative potential. Treatment of cells with PMP increased expression of mature endothelial cell markers on the progenitor cells and promoted both EC adhesion and paracrine activity, leading to improved endothelial healing. Exposure of endothelial progenitor cells to PMP amplified key functions of those cells including recruitment and migration to sites of injury, differentiation, and release of angiogenic factors (Barry et al. 1998; Diamant et al. 2004).

Clinical relevant application of PMP was demonstrated using a model of myocardial infarction in rats. Ischemia was created by left anterior descending coronary artery ligation followed by injection intramuscularly of PMP into the peri-ischemic region. PMP treatment resulted in a marked increase in the amount of capillaries in the ischemic myocardium compared to phosphate-buffered saline (PBS)-treated animals (Brill et al. 2005).

We studied the effect of PMP on 3D neural stem cell cultures to evaluate their potential pro-regenerative effect on neurogenesis and angiogenesis in the ischemic brain. We explored the mechanisms by which bioactive factors contained within PMP improve viability, proliferation, and differentiation of neural cells in culture.

Materials and Methods

PMP Preparation

PMP were produced from platelets obtained from healthy volunteers according to institutional Blood Bank regulations and with the approval of the institutional ethics committee. Outdated platelets portions were obtained from Blood Bank, and leukocytes were removed using PLX-5A leukocyte-

reducing filter (Asahi Kasei Medical Co, Ltd., Tokyo, Japan). Platelets were pelleted at $750\times g$ in the presence of 5 mM of citric acid, resuspended in 0.5 ml PBS (with Ca, Mg), and thrombin (2 U/ml) was added. After 5 min of incubation, platelet aggregates were removed along with thrombin residues and microparticles (including exosomes) were isolated by supernatant centrifugation at $100,000\times g$ for 1 h at 4°C. PMP were identified by flow cytometry using CD41 antibodies. The total protein amount in the obtained PMP was determined using the Bradford method.

For all the experiments, 10–100 $\mu\text{g/ml}$ PMP was used. According to our unpublished data, this amount of PMP contains about 0.1–1 pg/ml of VEGF and FGF and 1–10 pg/ml of PDGF. Since the PMP are from a human source, as a positive control we used recombinant human growth factors.

Growth of Mouse Neurospheres

Mouse embryos were collected at E13.5 from pregnant CD1 mice. The brain was removed under a dissection microscope (MZ6 Leica, Germany). Following removal of the meninges, the tissue was minced, digested in 0.025% Trypsin–EDTA solution (Gibco/BRL, Scotland) at 37°C for 5 min, and the enzymatic reaction was stopped by adding Trypsin inhibitor (Sigma Aldrich, Germany). Cell/tissue pellets were collected by centrifuging and then dissociated mechanically to yield a single cell suspension in serum-free DMEM/F12 medium (Biological Industries, Israel), containing B27 (without retinoic acid), L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 $\mu\text{g/ml}$). The cells were plated at a density of 5×10^6 cells in uncoated flasks and supplemented daily with 10 ng/ml of basic fibroblast growth factor (FGF2, R&D Minneapolis, MN) for 3 days. Spheres formed within a few days and were grown for 5 days with 10 ng/ml FGF2, VEGF (R&D, Minneapolis, MN)—the optimal concentration according to the literature (Einstein et al. 2003; Liu et al. 2011) or different doses of PMP—before collecting them for evaluation of their proliferation. Neurospheres size was analyzed by measuring sphere diameter under an inverted microscope. To further evaluate the role of specific factors contained within PMP on neurospheres size, we cultured neurospheres with PMP as detailed above and added blocking antibodies (0.5 $\mu\text{g/ml}$) to either human FGF2, PDGF, VEGF (R&D, Minneapolis, MN), or platelet factor 4 (PeproTech, Rocky Hill, NJ) and then analyzed sphere size.

Cell Viability Assay

We set up 96-well assay plates containing neurospheres in culture medium with different treatments (as described above) and added test compounds to appropriate wells so the final volume was 100 μl in each well. Cells were incubated for 4 h

at 37°C with 20 μ l of Cell Titer-Blue® Reagent in each well (Promega, Madison, WI). Finally, fluorescence was recorded at a 560/590-nm wavelength as per the manufacturer instructions.

FACS Analysis

Cells ($0.5 \times 10^6/10$ ml) were centrifuged at $450 \times g$ for 6 min, washed with cold PBS, and resuspended in 300 μ l PBS. The cells were fixed with 5 ml methanol and left at -20°C for at least 1 h. The cells were then centrifuged at $800 \times g$ for 5 min, resuspended in 100 μ l PBS and diluted to a final volume of 1 ml with PBS. Cells were incubated on ice for an additional 30 min, centrifuged at $800 \times g$ for 5 min and resuspended in 0.5 ml PBS. RNase (50 $\mu\text{g/ml}$) and propidium iodide (PI, 5 $\mu\text{g/ml}$; Sigma, Germany) were added to the cell samples which were FACS-analyzed for DNA content as a function of cell number. All centrifugations were carried out at 4°C. Samples were analyzed with a FACScan (Becton Dickinson, San Jose, CA) using the LYSYS II program.

Immunofluorescent Staining of NSCs

For characterization of cell differentiation, floating spheres were adhered to 35-mm tissue culture dishes coated with 10 $\mu\text{g/ml}$ poly-D-lysine and 5 $\mu\text{g/ml}$ fibronectin (Sigma, Germany) after 1 or 5 days of differentiation following withdrawal of treatments. Primary antibodies included: mouse anti-*nestin* (1:100; Chemicon, Billerica, MA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:200; Dako, Denmark), and mouse anti-neuronal class III β -tubulin (TUJ1, 1:500; Covance, France). Alexa Fluor antibodies were used for counterstaining (1:200; Invitrogen, England).

Electrophoresis and Immunoblotting

NSC cultures in 6-well plates were terminated with an ice-cold PBS rinse and addition of 100–150 μ l of cell lysis buffer 920 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , and 1 $\mu\text{g/ml}$ leupeptin (Cell Signaling Technology, Beverly, MA) with PMSF and protease inhibitors (Sigma, Germany). Cells were allowed to lyse on ice for 15 min then they were mixed, mechanically disrupted by pipetting, and transferred to chilled 1.5 ml Eppendorf tubes. Samples were centrifuged at 12,000 rpm for 20 min at 4°C; the total protein in each supernatant was quantified by the Bradford protein assay, and the lysates were stored at -80°C . Protein (2 μg) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride microporous membrane (Millipore, Billerica, MA). The

membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 followed by overnight incubation with primary antibodies for Phospho-Akt (Ser473), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), p44/42 MAPK (ERK1/2) rabbit mAb (1:1,000; Cell Signaling, Danvers, MA). After several washes, peroxidase-conjugated goat anti-rabbit IgG (Jackson, Baltimore, MD) was added and incubated for 1 h. The membrane was washed, and specific bands were visualized using an ECL kit (Biological Industries, Israel). The total protein content was normalized using rabbit anti-fibrillar antibodies (1:10,000; Abcam, England).

Statistical Analysis

Evaluations were performed by an examiner blinded to the experimental group. An analysis was performed with the SigmaStat software package (Systat, Richmond, CA, USA). Data are presented as mean \pm SE (SEM) as indicated in the legends. Values were compared using one-way analysis of variance followed by Bonferroni correction for multiple comparisons. *P* values < 0.05 were considered significant for all comparisons.

Results

PMP Increase NSC Proliferation and Survival

Neurospheres were grown in the presence of PMP containing medium, and their size was compared to that seen in spheres grown in control medium or in medium supplemented with FGF2 or VEGF. PMP treatment led to an increase in neurosphere size (Fig. 1) which was similar to that observed for spheres treated with VEGF or FGF2. In order to elucidate which factors within PMP mediate these effects on neurosphere size, we cultured neurospheres in the presence of PMP in combination with blocking antibodies specific to human FGF2, VEGF, PDGF, and platelet factor 4 (PF4). The results (Fig. 1) show that blocking FGF2, VEGF, or PDGF but not PF4 significantly inhibited the effects of PMP on sphere size. However, blocking of each individual growth factor did not completely block the effects of PMP on sphere growth suggesting an additive effect for at least FGF2, VEGF, and PDGF.

PMP Increase NSC Vitality and Decrease Cells Mortality

We analyzed endogenous neural stem cell (eNSC) survival by performing flow cytometry with propidium iodide (Fig. 2a). These results show that treatment with PMP led to a dose-dependent reduction in cell death. To substantiate these results, we used the cell titer blue commercial assay for measuring live cells in culture. Again, an increase in the

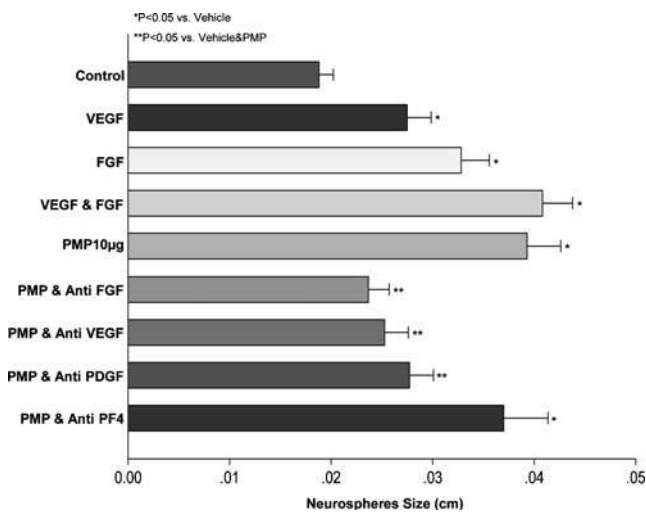


Fig. 1 PMP increase cell proliferation and survival. Neural stem cells obtained from E13.5 mice were grown as neurospheres. Figure 1 is a bar graph showing neurosphere size. Treatment with PMP significantly increased neurosphere size compared with vehicle, similarly to FGF2 and VEGF. Specific blockers to FGF2, VEGF, PDGF but not PF4 resulted in reduction in neurosphere size. The results are expressed as mean \pm SEM of three to five individual experiments

percentage of living cells was observed following treatment with PMP (Fig. 2b).

PMP Increase Neuronal Differentiation

We studied the effects of PMP treatment on neural stem cell (NSC) differentiation and found that PMP increased the percentage of differentiated neurons (TUJ1⁺) and astrocytes (GFAP⁺) and also increased the absolute number of newborn neurons and glia compared with untreated cells (Fig. 3). These effects were similar to the effects seen when either FGF2 or VEGF alone were used as positive controls.

PMP Increase Akt and ERK Phosphorylation

To study the signaling mechanisms involved, we tested whether PMP induce changes in the levels of phospho ERK and phospho Akt, molecules that are central to cell survival and proliferation. Our results show a dose dependent increase in pERK and pAkt following treatment with PMP. Of note, the effect of PMP treatment was larger than the effects of FGF2 or VEGF alone (Fig. 4).

Discussion

Stroke is a leading cause of chronic disability and mortality and therapies aimed at reducing this morbidity are currently limited. Repair mechanisms based on the proliferation of endogenous cells have been identified in ischemic brain

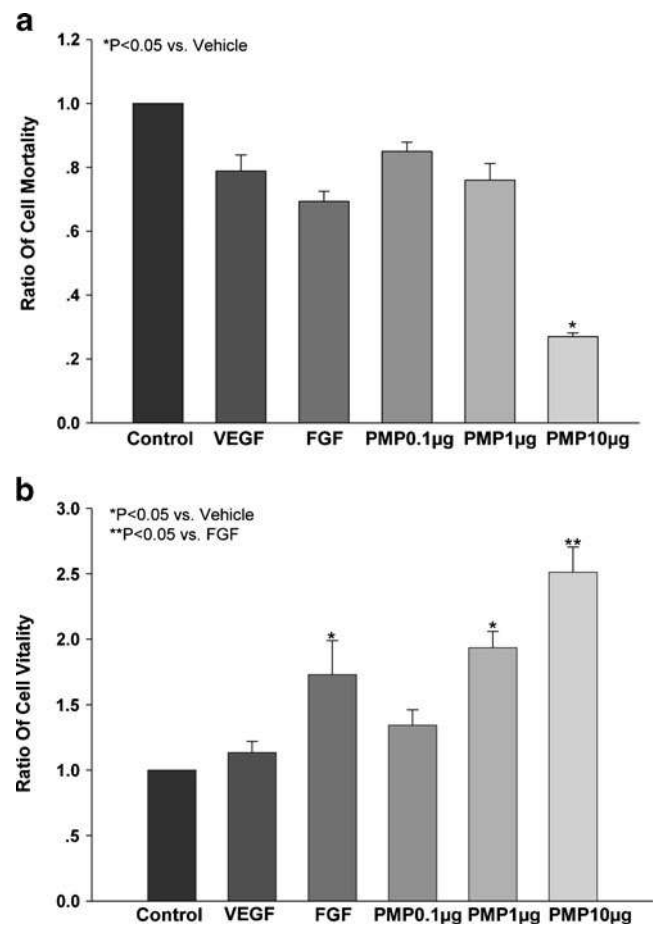


Fig. 2 PMP increase NSC vitality and decrease cells' mortality. Neural stem cells were grown as neurospheres and exposed to different treatments. Cell mortality was studied with FACS sorting according to the percentage of cells in sub-G1/GO phase (a). Note that PMPs reduced cell mortality in a dose-dependent manner and that the effect was larger than that observed with FGF or VEGF (a). Cell vitality was studied with the Cell Titer-Blue[®] Reagent as described in the "Materials and Methods" section, and survival was quantified (b). Note that PMPs increased cell vitality in a dose-dependent manner and that the effect was larger than that observed with FGF or VEGF (b). The results are expressed as mean \pm SEM of three to five individual experiments

regions suggesting that precursors at the ventricular surface proliferate, migrate to sites of injury and differentiate into neurons (Nakatomi et al. 2002; Parent 2003; Leker et al. 2007). Stimulation of endogenous repair mechanisms based on eNSC appears to be a promising novel therapeutic option for brain ischemic related diseases such as stroke (Ohab et al. 2006).

We hypothesize that the functional benefits obtained from the presence of newborn cells can be further enhanced by increasing the survival rates, migration, and differentiation potential of these cells.

The rationale behind this work was based on the observations that PMP can maintain a natural healing environment based on their endogenous inventory of trophic and mitogenic factors that are produced and secreted during

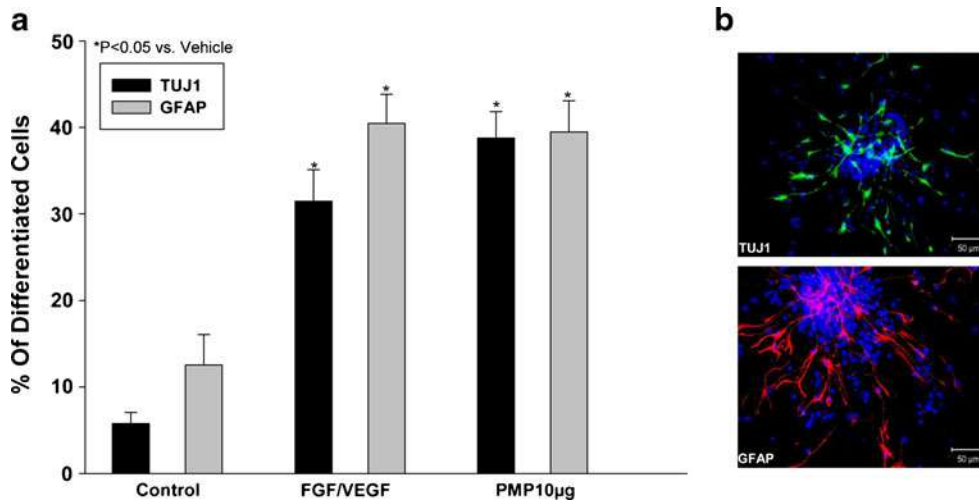


Fig. 3 PMP increase neuronal differentiation. Neural stem cells were grown as neurospheres and adhered for characterization cell differentiation. FGF2, VEGF, and PMP led to a significant increase in NSC differentiation into neurons or glia (a). Shown are photomicrographs of

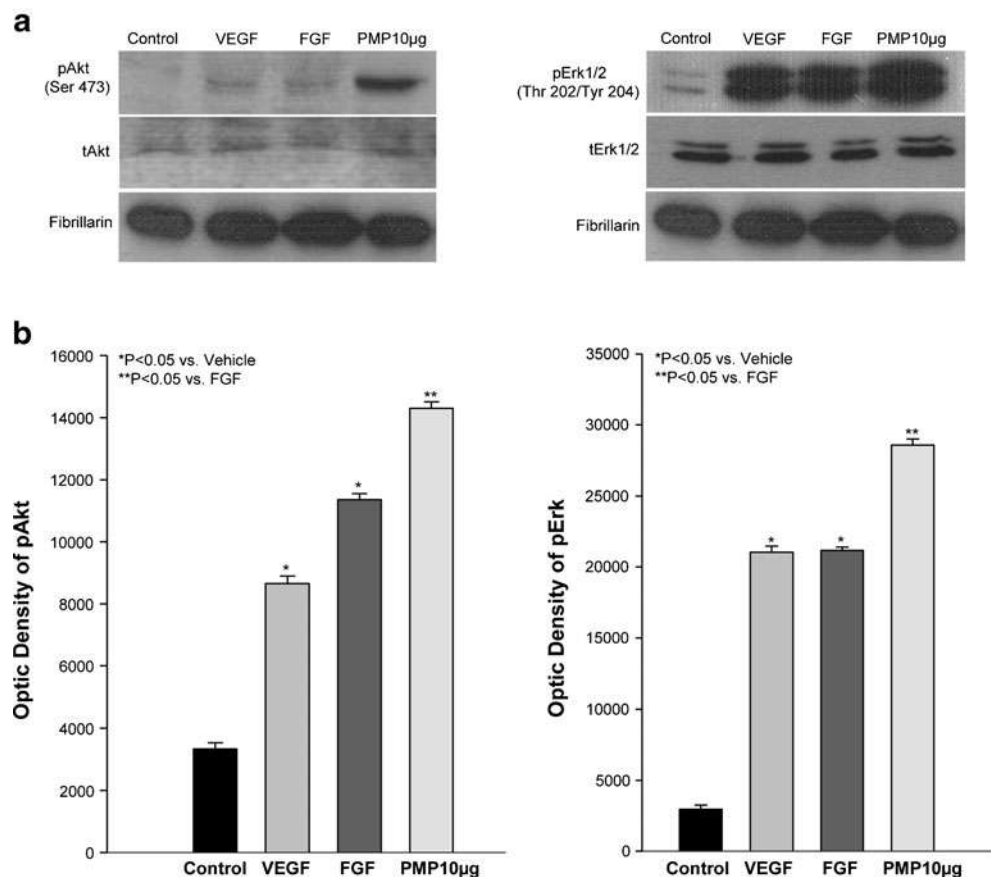
neurosphere cell differentiation into TUJ1⁺ neurons (green) and GFAP⁺ astrocytes (red) after treatment with PMP. Nuclei are counterstained with DAPI (blue) (b). The results are expressed as mean ± SEM of three to five individual experiments

normal injury response. Local delivery of bioactive molecules has potential to improve brain tissue regeneration and stroke therapy. Instead of promoting cell-specific tissue healing using a single growth factor or highly specialized stem cells (Johe et al. 1996; Cameron et al. 1998; Forsberg-

Nilsson et al. 1998), we capitalized the multitude of healing factors encapsulated by PMP in order to increase survival, proliferation, and differentiation of neural stem cells.

The current study demonstrates that PMP increase NSC proliferation and survival and increase the differentiation

Fig. 4 PMP increase Akt and ERK phosphorylation. Western blot analysis showed increased pAkt and pERK (a) levels upon treatment with PMP. The cells were preincubated with varying factors for 20 min. Cell extracts were processed for phospho-Akt, phospho-ERK, and the total protein content was normalized using anti-fibrillarlin antibodies. Note, that the effect of PMP treatment was larger than the effects of FGF2 or VEGF alone (b). The results are expressed as mean ± SEM of three to five individual experiments



potential of eNSC to glia and neurons. These effects are mediated by the combination of FGF2, VEGF, and PDGF (and potentially other growth factors), since blocking the individual effects of these cytokines (but not those of PF4) led to partial reversal of the proliferative effects of PMP.

Downstream signaling of PMP effects seem to be related to the observed increments in pERK and pAkt which are both implicated in cell survival and proliferation. PMP are shed from platelets upon activation and contain pro-survival and trophic factors including FGF2, VEGF, BDNF, PDGF, PF4 and also the receptors necessary for induction of these responses including VEGFR1. In particular, FGF2 is a well-known mitogen for NSC, and VEGF is a mitogen as well as a pro-migration factor for both NSC and EPC (Vicario-Abejon et al. 1995; Wada et al. 2003; Brusselmans et al. 2005; Wang et al. 2007). Furthermore, BDNF and PDGF are important for cell migration and differentiation (Fujimura et al. 2002). Our data suggest that the effects of PMP are mediated by a number of these factors working in concert as the effects on sphere size were reduced but not abolished by the addition of specific blockers to FGF2, VEGF, or PDGF to the medium. Since each of these factors activates specific receptors on NSC and EPC, it is likely that they all contribute to the overall observed effect.

The successful identification of factors governing neural progenitor cell proliferation, survival, migration, and differentiation will allow the development of interventions to manipulate progenitor cell fate after brain ischemia.

An attractive method for treating stroke would be stimulating repair mechanisms based on endogenous neural stem cells applied in situ using a minimally invasive procedure (Thored et al. 2006).

Toward this goal, we propose to evaluate the use of the neurogenic and angiogenic factors encapsulated in PMP. PMP paracrine factors have been shown to increase the number of blood vessels and proliferating cells in the ischemic heart (Brill et al. 2005), and this approach avoids the complex ethical and biological issues involved with the isolation and use of allogeneic embryonic stem cells or immortalized cell lines. This type of autologous therapy, if proven useful in vivo, may be an alternative to the economically burdensome recombinant growth factor approaches.

Taken together, our data show that treatment with PMP leads to an increase in NSC proliferation and survival, and to an increase in the absolute number of newborn neurons. Individual blocking of FGF2, VEGF, or PDGF reduces but does not completely abolish these effects suggesting that all these factors take part in mediating these effects.

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