Platelet microparticles induce angiogenesis *in vitro*

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Summary

Platelet microparticles (PMP) are endogenous substances generated during the coagulation process in a hypercoagulable state. This study demonstrated that PMP promote the proliferation and survival, migration, and tube formation in human umbilical vein endothelial cells (HUVEC). Heat-treated PMP did not significantly decrease the angiogenic activity in HUVEC compared with that of the untreated PMP. Meanwhile when PMP were treated with activated charcoal, a procedure known to remove the lipid growth factors, the angiogenic activity was significantly reduced. These results suggest that the lipid component(s) of the PMP may be major active factor(s) and that protein component(s) may be minor contributor(s). PMP were also shown to augment endothelial progenitor cell differentiation in peripheral blood mononuclear cells. In addition, PMP-stimulated proliferation, chemotaxis and tube formation of the HUVEC was mediated via the Pertussis toxin-sensitive G protein, extracellular signal-regulated kinase and the phosphoinositide 3-kinase pathway. Herein, a new action of PMP was demonstrated to be a potent angiogenic stimulator. It is expected that in pathological states such as a growing tumour, PMP shed from the circulating platelets may reach adequate concentrations and that the elevated levels of PMP could contribute to florid formation of new blood vessels.

Keywords: platelet microparticles, angiogenesis, proliferation, chemotaxis, tube formation.

Platelets are characteristically activated at the sites of injury and shed vesicular particles called platelet microparticles (PMP) (Horstman & Ahn, 1999). PMP have been shown to play a role in the normal haemostatic response to vascular injury because they are rich in membrane receptors for coagulation factors and provide a catalytic surface for the prothrombinase reaction (Sims *et al*, 1989). With the exception of the procoagulative properties, the functional importance of PMP *in vivo* has not been well defined. But recently there have been several reports suggesting that PMP play biological roles other than haemostasis *in vivo*. It was reported that PMP could stimulate the proliferation of smooth muscle cells (Weber *et al*, 2000) and haematopoietic cells (Baj-Krzyworzeka *et al*, 2002). At the molecular level, it was proved that PMP induced cyclooxygenase-2 expression in endothelial cells and monocytes via a protein kinase C/mitogen-activated protein kinase (MAPK)-dependent pathway (Barry *et al*, 1999). There was also an interesting report suggesting that PMP may attach to target cells and alter their binding characteristics, which was proved by improved homing of the haematopoietic stem cells after adding PMP to the circulation (Janowska-Wieczorek *et al*, 2001).

It seems quite reasonable that, on account of the haemostatic properties of PMP, elevated PMP levels have been detected in patients with disseminated intravascular coagulation, pulmonary embolism, coronary artery disease, transient ischaemic attacks and several other thrombotic diseases (Miyamoto *et al*, 1998). But in addition to these hypercoagulable states, we recently observed that plasma levels of PMP were markedly increased in patients with gastric cancer, particularly those with advanced disease (Kim *et al*, 2003). Interestingly, the PMP levels had a strong correlation with the levels of angiogenic factors, which suggest a possible role of PMP in angiogenesis.

The functional importance of platelets in angiogenesis has been proposed (Pinedo *et al*, 1998; Browder, 2003). It was reported that platelets can stimulate the endothelial cells to proliferate, migrate and affect their morphology (Pipili-Synetos *et al*, 1998), and are implicated in angiogenesis and tumour metastasis (Honn *et al*, 1992; Pinedo *et al*, 1998). On

the main functional molecules of these angiogenic effects, fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) were proposed to be the main contributors for the proliferation of the endothelial cells (Pintucci *et al*, 2002). But a recent study demonstrated that platelet-released phospholipids, such as sphingosine 1-phosphate (S1P), lysophosphatidic acid and phosphatidate, induce endothelial cell migration and tube formation (English *et al*, 2001). In spite of this well-known contribution of platelets to angiogenesis, no reports have described the role of PMP in angiogenesis.

Emerging data suggest that, in addition to the sprouting and co-option of neighbouring pre-existing vessels, angiogenesis is supported by the mobilization and functional incorporation of endothelial progenitor cells (EPC) to new blood vessel formation such as ischaemic tissues or growing tumours (Rafii *et al*, 2002). Haematopoietic/angiogenic cytokines such as VEGF and granulocyte colony stimulating factor (G-CSF) have been reported to augment EPC levels both *in vivo* and *in vitro* (Asahara *et al*, 1999; Takahashi *et al*, 1999). As, in our previous study, the level of PMP was closely correlated with several angiogenic factors, it is a reasonable assumption that PMP might have the potential to promote the EPC levels.

In this study, we showed that PMP isolated from the peripheral blood of normal volunteers promoted proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro*. We also showed that PMP augmented the EPC levels *in vitro*. Then we tracked down the active component in PMP and showed that lipid components of PMP were the dominant mediators of PMP-induced angiogenesis by the neutralization assays using anti-VEGF antibody and anti-FGF-2 antibody, heat inactivation and charcoal treatment. Finally we showed that these effects of PMP on endothelial cells depend on signal transduction downstream to cell surface receptors by an inhibition assay using the various kinase inhibitors.

Methods

Preparation of platelet microparticles

Platelet microparticles were prepared as described earlier (Barry *et al*, 1999). Platelet-rich plasma was prepared from the citrated blood of healthy donors by centrifugation at 150 ***g*** for 15 min. The supernatant was centrifuged at 500 ***g*** for 10 min, and the pellet was suspended in phosphate-buffered saline (PBS). The washed platelets were stimulated for 15 min with 1 U/ml thrombin, and the PMP were separated from the activated platelets by centrifugation at 1000 ***g*** for 20 min. From the resulting supernatant, the PMP were then pelleted by centrifugation at 17 000 ***g*** for 1 h. The PMP were washed with PBS and then resuspended in PBS. The PMP were quantified using flow cytometry (Epics XL; Beckman-Coulter, Fullerton, CA, USA) as previously described (Kim *et al*, 2002). The protein concentration was measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). A 5×10^6/ml PMP was equivalent to 30 μg/ml of the protein. The PMP were extracted with 1·5% activated charcoal (Sigma, St Louis, MO, USA) at 4°C for 24 h to remove the biologically active lipids (Lee *et al*, 1998). Where indicated, the PMP were heat-treated at 65°C for 60 min to eliminate the protein components.

Endothelial cell culture

HUVEC were obtained from Clonetics Corp. (Walkersville, MD, USA) and grown in endothelial growth media (EGM-2) MV SingleQuots (Clonetics) supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin (Gibco, Grand Island, NY, USA). The cells between passages 3 and 6 were used in all the experiments.

Proliferation assay

The HUVEC were starved of serum and growth factor overnight and then incubated for an additional 48 h with or without (control) the PMP or VEGF (BD Biosciences, Bedford, MA, USA). The effect of the PMP on cell proliferation was assayed using a validated colorimetric proliferation assay (CellTiter 96 Aqueous One Solution Cell Proliferation; Promega, Madison, WI, USA) according to the manufacturer's protocol. In subsequent studies, either 50 μg/ml of anti-VEGF or anti-FGF-2 (R & D Systems, Minneapolis, MN, USA), either alone or in combination, was added to the medium containing the PMP. For the inhibition studies, the HUVEC were incubated with PMP and the respective inhibitors (wortmannin, PD98059, Pertussis toxin; Sigma) for 48 h.

Apoptosis assays

The apoptosis of HUVEC was induced by serum deprivation following the method described previously (Araki *et al*, 1990). Annexin-V-fluorescein isothiocyanate (FITC) conjugate (Dinona, Seoul, Korea) was used to detect any apoptosis. The HUVEC were cultured under each condition (complete endothelial cell growth medium, serum-free medium, PMP, or VEGF) for 36 h after which the HUVEC were detached with trypsin, and incubated with the annexin-V-FITC reagent according to the manufacturer, which were then analysed by using a EPICS XL cytometer. Each specific apoptosis was calculated as given: (% experimental apoptosis−% complete endothelial growth medium)/(100−% complete endothelial growth medium).

Chemotaxis assay

The chemotactic motility was assayed using a Transwell chamber with an 8 μm-pore polycarbonate filter (Costar Corning, Cambridge, MA, USA). Briefly, the lower surface of the filter was coated with 0·1% fibronectin. Serum free Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco)

containing either PMP or VEGF (as a positive control for chemoattractant) was added to the lower wells. The HUVEC (1×10^5/100 μl) were placed in the upper wells and the chamber was then incubated (37°C, 5% CO_2, for 2 h). The inhibitors were then added to the cells for 30 min at room temperature prior to seeding. The filters were stained with a Diff-Quik Stain Set (Dade Behring, Dudingen, Switzerland). The non-migrating cells on the upper surface of the filters were removed by wiping with a cotton swab, and the cells that migrated across the filter towards the lower surface were counted using optical microscopy. Ten fields (×400) were counted for each assay.

In vitro endothelial cell morphogenesis assay

In order to investigate the *in vitro* angiogenic activity, the endothelial cell differentiation into the capillary-like tube structures was monitored, as described previously (Gho *et al*, 1999). HUVEC (40 000 cells), in 1 ml of serum free medium with various concentrations of PMP or VEGF, were added to each well, coated with Matrigel (BD Biosciences). The inhibitors were added to the cells for 30 min at room temperature prior to seeding. After 5 h incubation at 37°C, the capillary-like structures were quantified over 20 randomly selected fields (100) using an inverted Olympus IX70 microscope (Olympus, Tokyo, Japan).

EPC culture

Peripheral blood was drawn from human volunteers. The peripheral blood mononuclear cells (PBMC) were isolated using density-gradient centrifugation with Ficoll–Paque Plus (Amersham Biosciences, Piscataway, NJ, USA). The isolated PBMC were then resuspended in EGM-2 MV SingleQuots (Clonetics) and placed on six-well culture plates coated with fibronectin (BD Biosciences). In order to confirm the EPC phenotype, the adherent cells were incubated with a DiI-acetylated low-density lipoprotein (DiLDL; Molecular Probes, Eugene, OR, USA) for 1 h and after fixation, they were incubated with FITC-labelled *Ulex europaeus* agglutinin I (lectin; Sigma) for 1 h. The cells were observed using inverted fluorescent microscopy. EPC, which are recognized as attached, spindle-shaped cells (Asahara *et al*, 1999), were identified by the co-expression of DiLDL and lectin. All the experiments were performed using the day 7 EPC culture. All the cells were growth arrested for 18 h in serum-free medium. The EPC were stimulated with PMP (final concentration 5×10^6/ml), VEGF or the serum-free medium only for 72 h. Thirty randomly selected fields (×200) from two individual wells were examined in each sample and the number of EPC was counted.

Statistics

Analyses of all of the test samples were performed in triplicate and repeated at least twice. The data is presented as a mean ± standard error of mean. Comparisons between two groups of means were assessed by an unpaired Student's *t*-test. Two-sided *P*-values $<0{\cdot}05$ were considered to be significant. The analysis was carried out using the Statistical Package for the Social Sciences (SPSS).

Results

PMP stimulate proliferation of human endothelial cell and inhibit its apoptosis

The effect of PMP on the proliferation of HUVEC was measured by proliferation assay after incubation for 48 h. In this assay, PMP induced proliferation of HUVEC in a dose-dependent manner. At the concentration of 10×10^6/ml, PMP nearly doubled the proliferation rate of HUVEC, which was comparable with the effect of VEGF at 50 ng/ml (Fig. 1A).

In search of the active components in PMP, we performed a series of neutralization assays, including heat treatment, neutralization of VEGF and FGF-2 and charcoal treatment. Heat treatment reduced, but did not eradicate the effect of PMP on endothelial cell proliferation, which suggested that a fraction of active component might be a protein (Fig. 1B). Therefore specific neutralizing antibodies to either VEGF or FGF-2, which are very well-known growth factors of HUVEC (Pintucci *et al*, 2002), were tested to determine whether they could inhibit the effect of PMP on HUVEC proliferation. The combination of these two antibodies significantly inhibited the effect of PMP, which was nearly equivalent to the heat treatment, whereas inhibition of either VEGF or FGF alone had no effect on the PMP-stimulated HUVEC proliferation (Fig. 1B). Thereafter, we checked whether the lipid growth factors played a major role in HUVEC proliferation. To remove only lipid growth factors while preserving the protein growth factor including VEGF and FGF-2, we treated PMP with activated charcoal as described previously (English *et al*, 2000). With this treatment, the activity of PMP on HUVEC proliferation was significantly depleted (Fig. 1B). This result suggested that PMP contained both protein growth factors, such as VEGF and FGF-2, and lipid growth factors.

We next examined whether PMP could inhibit the apoptosis of HUVEC induced by serum deprivation. The addition of PMP successfully prevented apoptosis (Fig. 2) while VEGF provided no statistically meaningful protection, even at the concentration of 50 ng/ml, at which it nearly doubled the proliferation rate of HUVEC as described above (Fig. 1A). This protective effect of PMP was lost when PMP were treated with activated charcoal. Heat treatment showed no statistically meaningful changes on the protective property of PMP. All these results suggested that the major survival factor(s) might be lipid component(s) that can be absorbed by activated charcoal.

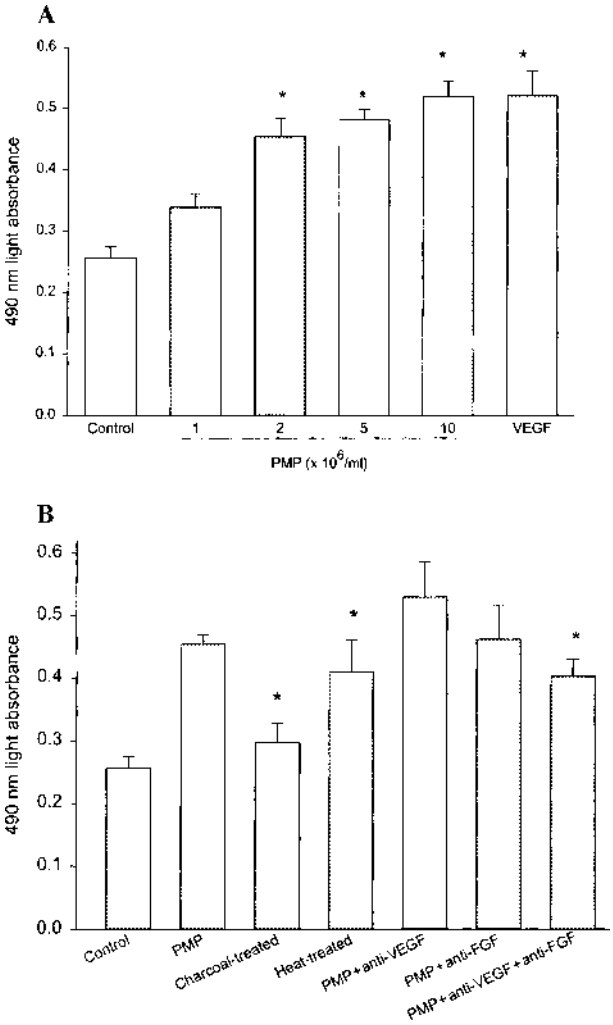


Fig 1. Platelet microparticles (PMP) stimulated human umbilical vein endothelial cells (HUVEC) proliferation. (A) After 48 h of incubation, PMP stimulated HUVEC proliferation in a moderate dose-dependent manner (*$P < 0{\cdot}05$ vs. serum-free control). (B) PMP, heat-treated PMP, and charcoal-treated PMP at a 5×10^6/ml concentration was added to the HUVEC for 48 h. The effect of PMP was significantly depleted by the charcoal-treatment (*$P < 0{\cdot}05$ vs. PMP). Heat treatment decreased the endothelial cell proliferation (*$P < 0{\cdot}05$ vs. PMP) but still induced the proliferation more than that of the medium alone. The inhibition of either VEGF or FGF alone had no effect on PMP-stimulated HUVEC proliferation. Neutralization of both VEGF and FGF-2 inhibited HUVEC proliferation moderately (*$P < 0{\cdot}05$ vs. PMP).

PMP promoted chemotaxis and tube formation of endothelial cells

The effect of PMP on endothelial cell migration was investigated using a modified Boyden chamber assay because the directed chemotactic migration of the endothelial cells through the extracellular matrix is essential for neovascularization. PMP profoundly enhanced cell migration in a dose-dependent manner (Fig. 3A). The migratory activity at 5×10^6/ml PMP was approximately two times that of the control, which was higher than that of VEGF at 50 ng/ml. The migratory activity was completely depleted by treating the PMP with activated charcoal. Heat treatment only slightly decreased the chemotactic activity of the PMP. This suggests that a lipid component(s) plays a major role in endothelial cell migration.

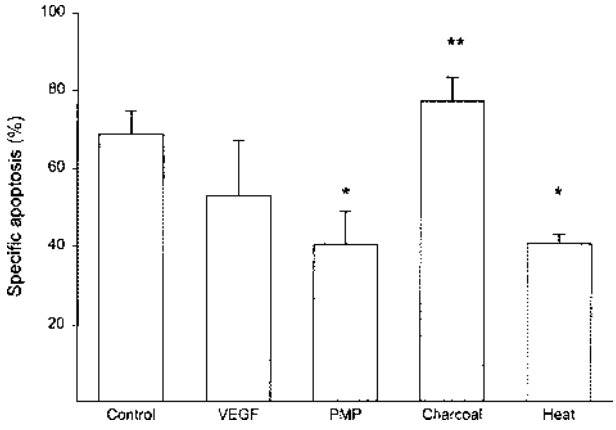


Fig 2. Platelet microparticles (PMP) protected human umbilical vein endothelial cells (HUVEC) from apoptosis. The addition of PMP (5×10^6/ml) rescued HUVEC from the apoptosis induced by serum deprivation (*$P < 0{\cdot}05$ vs. control). VEGF (50 ng/ml) did not significantly decrease HUVEC apoptosis. Charcoal treatment of PMP (5×10^6/ml) abrogated the PMP-induced survival (**$P < 0{\cdot}05$ vs. PMP). Heat-treated PMP (5×10^6/ml) could still rescue HUVEC from apoptosis (*$P < 0{\cdot}05$ vs. control).

Then we tested whether PMP induced the morphogenic differentiation of HUVEC using a capillary-like tube formation assay (Grant *et al*, 1992). PMP stimulate tube formation of the HUVEC in a dose-dependent manner (Fig. 3B). The presence of PMP at 5×10^6/ml and 10×10^6/ml showed a significant 2·4- and 3·8-fold increase in the tube area over the control containing medium alone, respectively. VEGF (50 ng/ml) also stimulated a 2·5-fold increase in tube formation. The morphogenic activity was also completely depleted after treating the PMP with the activated charcoal but not by heat treatment. This indicated that the lipid component(s) extracted by the activated charcoal might be the major contributor(s).

PMP stimulated the growth of endothelial progenitor cells in vitro culture

The cultured PBMC of healthy volunteers showed attached, spindle-shaped cells. The EPC were characterized as being adherent cells that were doubly positive for DiLDL and lectin (Fig. 4). When these cells were stimulated with PMP for 72 h at the concentration of 5×10^6/ml, the number of EPC per area was twofold higher than that of the untreated control ($11{\cdot}3 \pm 0{\cdot}6/mm^2$ vs. $20{\cdot}6 \pm 2{\cdot}4/mm^2$, $P < 0{\cdot}05$). VEGF, a well-known angiogenic factor to augment the number of EPC (Asahara *et al*, 1999; Kalka *et al*, 2000), also stimulated EPC formation ($24{\cdot}3 \pm 4{\cdot}9/mm^2$, $P < 0{\cdot}05$) at the concentration of 50 ng/ml.

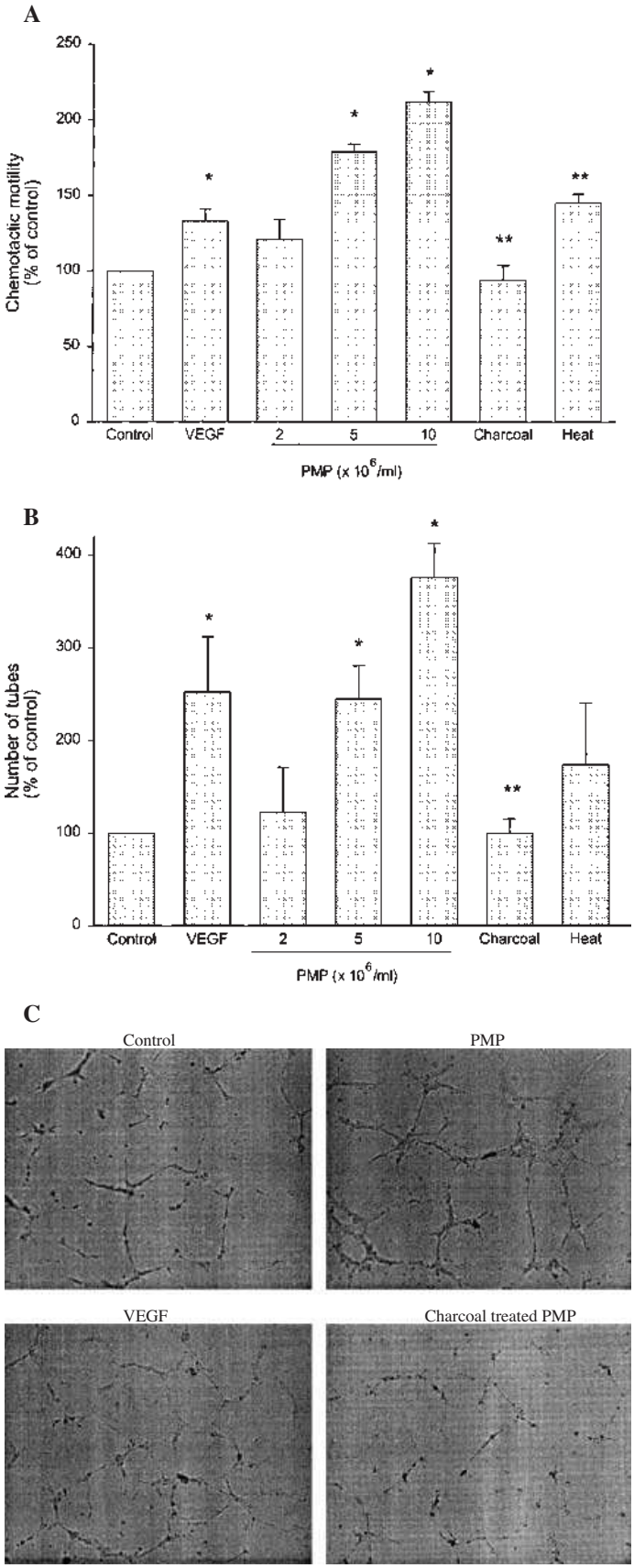
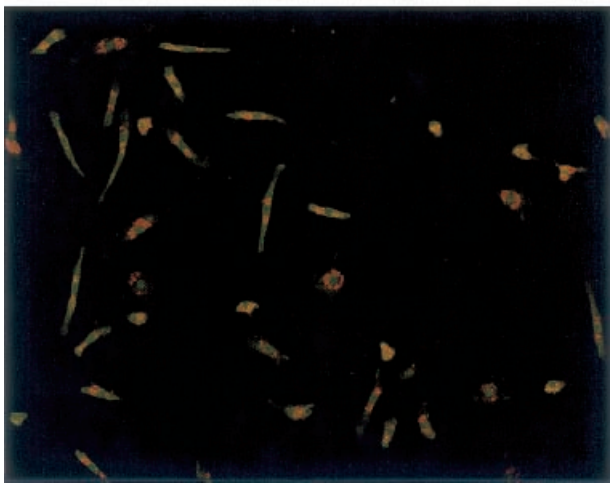


Fig 3. (A) Platelet microparticles (PMP) stimulated the migration of human umbilical vein endothelial cells (HUVEC). PMP profoundly enhanced cell migration in a dose-dependent manner (*$P < 0{\cdot}05$ vs. control). The migratory activity of PMP was completely depleted by treatment with activated charcoal (**$P < 0{\cdot}05$ vs. 5×10^6/ml PMP). Heat treatment slightly decreased the endothelial cell chemotactic activity of PMP, but still induced the migratory activity (**$P < 0{\cdot}05$ vs. 5×10^6/ml PMP). (B) PMP stimulated the tube formation of the HUVEC in a dose-dependent manner. The presence of PMP at 5×10^6/ml and 10×10^6/ml resulted in a significant, 2·4- and 3·8-fold, increase in the tube area (*$P < 0{\cdot}001$ vs. control). The morphogenic activity was completely depleted by treatment with activated charcoal (**$P < 0{\cdot}05$ vs. 5×10^6/ml PMP). Heat treatment did not significantly decrease endothelial cell migration. (C) Phase contrast micrographs (×200) after a 5 h incubation of HUVEC in a Matrigel-coated well with or without (control) 50 ng/ml vascular endothelial growth factor (VEGF) and PMP and charcoal-treated PMP.

Control



PMP

Fig 4. Representative fluorescence photomicrographs of endothelial progenitor cells (EPC). EPC were characterized as adherent cells doubly positive for DiLDL (red) and lectin (green).

PMP-stimulated proliferation, chemotaxis and tube formation of HUVEC could be inhibited by wortmannin (phosphoinositide 3-kinase inhibitor), PD98059 (extracellular signal-regulated kinases 1/2 inhibitor), and PTx (Gi protein inhibitor)

As VEGF was reported to induce angiogenesis by activating the MAPK/extracellular signal-regulated kinases (ERK) and phosphoinositide 3-kinase (PI3K)/Akt pathway via VEGF receptor 2 (VEGFR-2; Flk-1/KDR) (Rousseau *et al*, 1997) and S1P, a lipid angiogenic factor, was reported also to stimulate the same pathway via PTx-sensitive G*i* protein by binding to the endothelial differentiation gene (EDG) (Tamama & Okajima, 2002), wortmannin (PI3K inhibitor), PD98059 (ERK1/2 inhibitor), and PTx (G*i* protein inhibitor) were examined to determine if they could inhibit PMP-stimulated proliferation, chemotaxis and tube formation of the HUVEC. Treatment with 20 ng/ml PTx inhibited PMP-induced proliferation, chemotaxis and tube formation (Fig. 5). Wortmannin (10 nmol/l) markedly inhibited the PMP-induced proliferation, chemotaxis and tube formation (Fig. 5). PD98059 (10 μmol/l) inhibited the PMP-induced proliferation and migration (Fig. 5). However, HUVEC tube formation was not inhibited by 10 μmol/l PD98059 (Fig. 5).

Discussion

Under normal conditions, the angiogenesis process is tightly regulated in order to avoid neovascularization, and many control mechanisms have been suggested to be active (Hanahan & Folkman, 1996). The proliferative states, such as ischaemic disease, inflammation and cancer, are often accompanied by intense angiogenesis, which is a highly orchestrated process involving endothelial cell migration, proliferation and maturation. However, a mediator of angiogenesis at sites of injury and pathological angiogenesis is not yet well defined.

One of the new findings of this study was that PMP induced proliferation, chemotaxis and tube formation of endothelial cells *in vitro*. As platelets contain both inducers and inhibitors of angiogenesis that could be harnessed to modulate angiogenesis locally within the vasculature (Folkman *et al*, 2001), it was not certain whether the PMP shed from platelets function as an agonist or antagonist of angiogenesis. It is important to note that PMP plasma levels in cancer patients ($1–5 \times 10^6$/ml) (Kim *et al*, 2003) were closely related to the PMP concentration used in our assays (5×10^6/ml), which have been shown to be active for angiogenesis. Although this has not been confirmed *in vivo*, one would expect that in pathological states, such as a growing tumour, PMP shed from circulating platelets may reach adequate concentrations, and that the increased PMP levels could contribute to florid formation of new blood vessels.

We also, for the first time, demonstrated that PMP stimulated the growth of EPC *ex vivo*. It was reported that proangiogenic growth factors, such as VEGF (Asahara *et al*, 1999) and granulocyte-macrophage CSF (Takahashi *et al*, 1999) and 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor (statins) (Dimmeler *et al*, 2001), augmented EPC levels. The effect of 5×10^5/ml PMP appears to be at least as potent as that of 50 ng/ml VEGF. Related with this finding, PMP was recently reported to stimulate proliferation of haematopoietic cells and to increase the survival of human $CD34^+$ cells (Baj-Krzyworzeka *et al*, 2002). Potential candidates for this effect were suggested to be CD40L expressed on the surface of PMP or bioactive lipids (Amirkhosravi *et al*, 2002; Baj-Krzyworzeka *et al*, 2002). Therefore, it is a reasonable assumption that, PMP generated during coagulation at the sites of injury and wound repair or in pathological conditions like tumours are involved in neovascularization through augmenting the number of EPC.

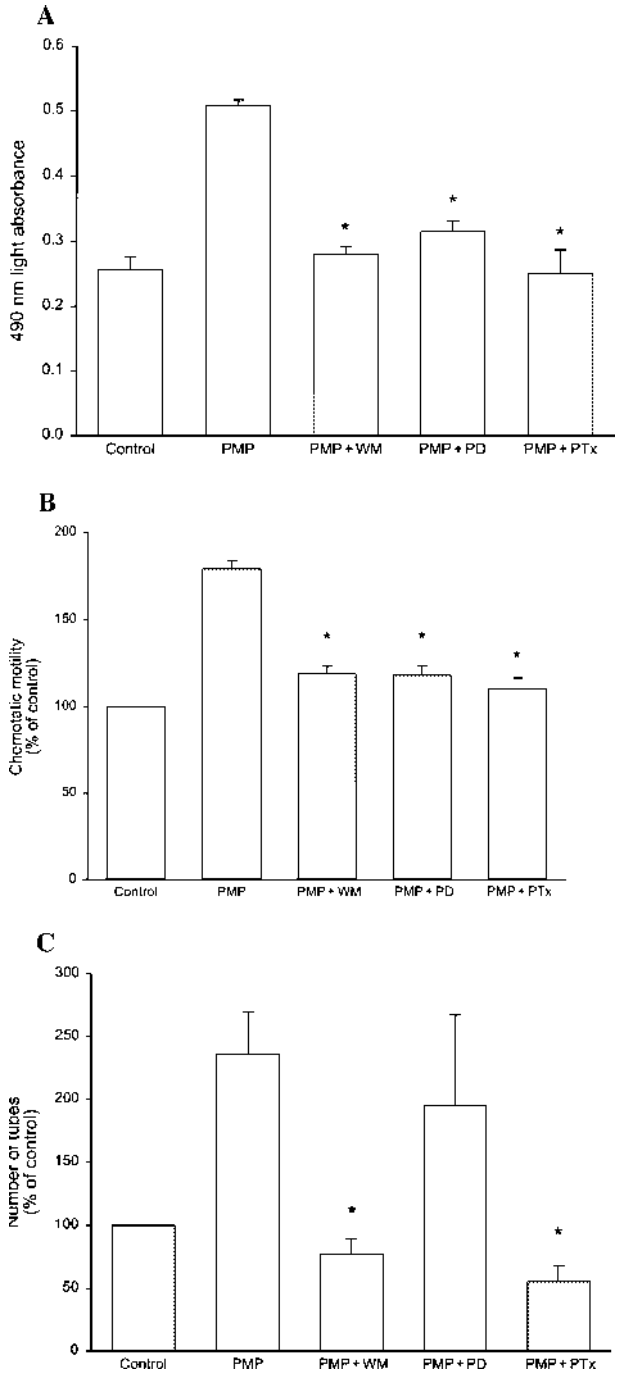


Fig 5. (A) human umbilical vein endothelial cells (HUVEC) were incubated in 5×10^6/ml platelet microparticles (PMP) with or without the presence of the respective inhibitors (WM, 10 nmol/l wortmannin; PD, 10 μmol/l PD98059; PTx, 20 ng/ml pertussis toxin) for 48 h. Wortmannin, PD98059 and PTx markedly inhibited HUVEC proliferation induced by PMP. (B) Treatment with wortmannin, PD98059 and PTx almost completely inhibited the PMP-induced chemotaxis, respectively. (C) Wortmannin and PTx markedly inhibited PMP-induced tube formation, but PD98059 did not show any effect (*$P < 0{\cdot}05$ vs. PMP).

In this study we also tried to define the active components involved in the angiogenic activities of PMP. Heat-treated PMP slightly decreased endothelial cell proliferation, which suggests that some of active factors may be proteins. As neutralization of both VEGF and FGF-2 moderately inhibited HUVEC proliferation, the cooperative effects of VEGF and FGF-2 might partly contribute to HUVEC proliferation. When we treated PMP with activated charcoal, a procedure known to remove lipid growth factors, the activity on HUVEC proliferation was significantly depleted. In an endothelial cell migration assay, both heat treatment and charcoal treatment significantly affected the activity of PMP, with the effect of the charcoal treatment being more drastic. In an assay to determine the role of PMP in the protection of HUVEC from apoptosis and tube formation, only the charcoal treatment significantly affected the activity of PMP. All these results suggested that lipid component(s) of PMP might play a major role in angiogenesis. In accordance with these results, a recent study demonstrated that platelet-released phospholipids induced endothelial cell migration and tube formation (English *et al*, 2001). It was also reported that lipid growth factors in serum were released from activated platelets, which was found to be remarkably heat stable and readily extracted with activated charcoal (English *et al*, 2000).

One of these platelet-derived lipid growth factors, S1P, has been shown to mediate cytoprotection, proliferation, migration and blood vessel formation via its receptors on the endothelial cells, EDG-1 and EDG-3, which was originally cloned as a phorbol 12-myristate 13 acetate-inducible early response gene in HUVEC (Tamama & Okajima, 2002). Previously it was reported that the EDG/G*i*/ERK pathway plays a dominant role in the S1P-induced proliferation and cytoprotection of endothelial cells, and that S1P-induced migration of endothelial cells has been shown to be sensitive to the inhibitors of the G*i* protein and PI3K/Akt (Kimura *et al*, 2000). In this context, it seemed possible that the effect of PMP on HUVEC was inhibited by G*i* protein and PI3K inhibitors (Fig. 5).

In summary, we report for the first time that PMP, at the level commonly found in patients with pathological conditions such as cancer, can stimulate human endothelial cells to proliferate, migrate and form tubes essential for angiogenesis, *in vitro*. The increased EPC levels also induced by PMP might augment this angiogenic environment. Both protein and lipid growth factors were involved but it seemed that the effects of the latter were greater. In this context, it might be possible to inhibit angiogenesis in pathological conditions, such as the tumour vasculature, by lowering the PMP level.

Acknowledgments

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References

Amirkhosravi, A., Amaya, M., Desai, H. & Francis, J.L. (2002) Platelet–CD40 ligand interaction with melanoma cell and monocyte CD40 enhances cellular procoagulant activity. *Blood Coagulation and Fibrinolysis*, **13,** 505–512.

Araki, S., Shimada, Y., Kaji, K. & Hayashi, H. (1990) Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. *Biochemical and Biophysical Research Communications*, **168,** 1194–1200.

Asahara, T., Takahashi, T., Masuda, H., Kalka, C., Chen, D., Iwaguro, H., Inai, Y., Silver, M. & Isner, J.M. (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO Journal*, **18,** 3964–3972.

Baj-Krzyworzeka, M., Majka, M., Pratico, D., Ratajczak, J., Vilaire, G., Kijowski, J., Reca, R., Janowska-Wieczorek, A. & Ratajczak, M.Z. (2002) Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells. *Experimental Hematology*, **30,** 450–459.

Barry, O.P., Kazanietz, M.G., Pratico, D. & FitzGerald, G.A. (1999) Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. *Journal of Biological Chemistry*, **274,** 7545–7556.

Browder, T. (2003) Angiogenesis and hemostasis. In: *Thrombosis and Hemorrhage* (ed. by J. Loscalzo & A.I. Schafer), pp. 220–235. Lippincott Williams & Wilkins, Philadelphia, PA.

Dimmeler, S., Aicher, A., Vasa, M., Mildner-Rihm, C., Adler, K., Tiemann, M., Rutten, H., Fichtlscherer, S., Martin, H. & Zeiher, A.M. (2001) HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *Journal of Clinical Investigation*, **108,** 391–397.

English, D., Welch, Z., Kovala, A.T., Harvey, K., Volpert, O.V., Brindley, D.N. & Garcia, J.G. (2000) Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *FASEB Journal*, **14,** 2255–2265.

English, D., Garcia, J.G. & Brindley, D.N. (2001) Platelet-released phospholipids link haemostasis and angiogenesis. *Cardiovascular Research*, **49,** 588–599.

Folkman, J., Browder, T. & Palmblad, J. (2001) Angiogenesis research: guidelines for translation to clinical application. *Thrombosis and Haemostasis*, **86,** 23–33.

Gho, Y.S., Kleinman, H.K. & Sosne, G. (1999) Angiogenic activity of human soluble intercellular adhesion molecule-1. *Cancer Research*, **59,** 5128–5132.

Grant, D.S., Kinsella, J.L., Fridman, R., Auerbach, R., Piasecki, B.A., Yamada, Y., Zain, M. & Kleinman, H.K. (1992) Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) in vitro and induction of angiogenic behavior in vivo. *Journal of Cellular Physiology*, **153,** 614–625.

Hanahan, D. & Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, **86,** 353–364.

Honn, K.V., Tang, D.G. & Chen, Y.Q. (1992) Platelets and cancer metastasis: more than an epiphenomenon. *Seminars in Thrombosis and Hemostasis*, **18,** 392–415.

Horstman, L.L. & Ahn, Y.S. (1999) Platelet microparticles: a wide-angle perspective. *Critical Reviews in Oncology/Hematology*, **30,** 111–142.

Janowska-Wieczorek, A., Majka, M., Kijowski, J., Baj-Krzyworzeka, M., Reca, R., Turner, A.R., Ratajczak, J., Emerson, S.G., Kowalska, M.A. & Ratajczak, M.Z. (2001) Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood*, **98,** 3143–3149.

Kalka, C., Masuda, H., Takahashi, T., Gordon, R., Tepper, O., Gravereaux, E., Pieczek, A., Iwaguro, H., Hayashi, S.I., Isner, J.M. & Asahara, T. (2000) Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circulation Research*, **86,** 1198–1202.

Kim, H.K., Song, K.S., Lee, E.S., Lee, Y.J., Park, Y.S., Lee, K.R. & Lee, S.N. (2002) Optimized flow cytometric assay for the measurement of platelet microparticles in plasma: pre-analytic and analytic considerations. *Blood Coagulation and Fibrinolysis*, **13,** 393–397.

Kim, H.K., Song, K.S., Park, Y.S., Kang, Y.H., Lee, Y.J., Lee, K.R., Ryu, K.W., Bae, J.M. & Kim, S. (2003) Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *European Journal of Cancer*, **39,** 184–191.

Kimura, T., Watanabe, T., Sato, K., Kon, J., Tomura, H., Tamama, K., Kuwabara, A., Kanda, T., Kobayashi, I., Ohta, H., Ui, M. & Okajima, F. (2000) Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochemical Journal*, **348,** 71–76.

Lee, M.J., Van Brocklyn, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S. & Hla, T. (1998) Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science*, **279,** 1552–1555.

Miyamoto, S., Marcinkiewicz, C., Edmunds, Jr, L.H. & Niewiarowski, S. (1998) Measurement of platelet microparticles during cardiopulmonary bypass by means of captured ELISA for GPIIb/IIIa. *Thrombosis and Haemostasis*, **80,** 225–230.

Pinedo, H.M., Verheul, H.M., D'Amato, R.J. & Folkman, J. (1998) Involvement of platelets in tumour angiogenesis? *Lancet*, **352,** 1775–1777.

Pintucci, G., Froum, S., Pinnell, J., Mignatti, P., Rafii, S. & Green, D. (2002) Trophic effects of platelets on cultured endothelial cells are mediated by platelet-associated fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). *Thrombosis and Haemostasis*, **88,** 834–842.

Pipili-Synetos, E., Papadimitriou, E. & Maragoudakis, M.E. (1998) Evidence that platelets promote tube formation by endothelial cells on matrigel. *British Journal of Pharmacology*, **125,** 1252–1257.

Rafii, S., Lyden, D., Benezra, R., Hattori, K. & Heissig, B. (2002) Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nature Reviews Cancer*, **2,** 826–835.

Rousseau, S., Houle, F., Landry, J. & Huot, J. (1997) p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene*, **15,** 2169–2177.

Sims, P.J., Wiedmer, T., Esmon, C.T., Weiss, H.J. & Shattil, S.J. (1989) Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *Journal of Biological Chemistry*, **264,** 17049–17057.

Takahashi, T., Kalka, C., Masuda, H., Chen, D., Silver, M., Kearney, M., Magner, M., Isner, J.M. & Asahara, T. (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nature Medicine*, **5,** 434–438.

Tamama, K. & Okajima, F. (2002) Sphingosine 1-phosphate signaling in atherosclerosis and vascular biology. *Current Opinion in Lipidology*, **13,** 489–495.

Weber, A., Koppen, H.O. & Schror, K. (2000) Platelet-derived microparticles stimulate coronary artery smooth muscle cell mitogenesis by a PDGF-independent mechanism. *Thrombosis Research*, **98,** 461–466.