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Epidermal growth factor released from platelet-rich plasma promotes endothelial cell proliferation *in vitro*

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Background and Objective: The therapeutic benefits of platelet-rich plasma (PRP) for the promotion of healing and regeneration of periodontal tissues are thought to result from enrichment in growth factors released from platelets. The aim of this study was to evaluate the effects of specific growth factors released from PRP on endothelial cell proliferation.

Material and Methods: The levels of vascular endothelial growth factor (VEGF), platelet-derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) in supernatants of calcium- and thrombin-activated PRP samples from five donors were quantified by enzyme-linked immunosorbent assay. Supernatants were treated with neutralizing antibodies specific to each growth factor, and the effects of these treatments on human umbilical vein endothelial cell (HUVEC) proliferation *in vitro* were determined. The effect of removing EGF from PRP supernatants with antibody-coated beads on HUVEC proliferation was also tested.

Results: Average concentrations of VEGF, PDGF-BB, bFGF and EGF in PRP supernatants were 189, 27,190, 39.5 and 513 pg/mL, respectively. The addition of EGF neutralizing antibodies to the PRP supernatants significantly reduced HUVEC proliferation (up to 40%), while such an inhibition was not observed following neutralization of the other growth factors. Removal of EGF from PRP supernatants by treatment with antibody-coated beads also resulted in a significant decrease in HUVEC proliferation. Recombinant EGF increased HUVEC proliferation *in vitro* in a dose-dependent manner.

Conclusion: This study showed that PRP supernatants are highly mitogenic for endothelial cells and provided evidence that this effect may be due, at least in part, to the presence of EGF. *In vivo* experiments are needed to confirm the roles of specific growth factors released from PRP in the healing of oral surgical and/or periodontal wounds.

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Periodontitis is a chronic inflammatory disease of bacterial etiology that is characterized by destruction of tooth-supporting tissues, including periodontal ligament and alveolar bone. Healing and regeneration of the periodontal tissues is a complex process that can be divided into four major steps, which overlap each other: haemostasis,

inflammation, cell proliferation and remodelling (1). In bone repair, the cell proliferation phase includes angiogenesis (formation of new blood vessels from existing ones) and osteogenesis. Angiogenesis represents an essential step in the hard and soft tissue repair processes (2), since blood vessels provide the oxygen and growth factors (GFs) needed for cell differentiation, migration and division. The pivotal role of angiogenesis in bone regeneration has been demonstrated in numerous studies by the fact that genetic, biochemical or mechanical alterations of blood vessel formation all perturb bone synthesis (3,4).

Rupture of blood vessels, as a result of injury or surgical trauma, releases platelets, which become activated and trigger the synthesis of pro-angiogenic factors that regulate a complex cascade of events leading to formation of new blood vessels (5). A variety of GFs, including vascular endothelial growth factor (VEGF), platelet-derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), are involved in the regulation of the angiogenic process, and all of them can be released from activated platelets (6,7).

Preparation of platelet-rich plasma samples (PRPs) from autologous blood represents a safe and efficient way to obtain high concentrations of GFs in a small volume of plasma. This technique was introduced in 1997 by Whitman *et al.* (8), based on the premise that an enrichment in GFs may be beneficial to wound healing. This hypothesis was reinforced by the publication of a landmark study by Marx *et al.* (9), indicating that the addition of PRPs to autogenous bone grafts leads to faster bone maturation rates and to better radiographic densities and bone volumes than the use of autogenous bone alone. Since then, PRPs have been successfully used in a variety of clinical applications to improve the formation of hard and soft tissues (10–14). However, controversies exist in the literature regarding the benefit of this procedure. While some authors have reported significant increases in bone regeneration, others did not observe any improvement (15–18). Several reviews have emphasized the need for additional research with the intention of characterizing PRPs in terms of GF content and to determine their physiological roles in wound healing (1,19–21). We have previously reported that PRP supernatants are highly mitogenic for endothelial cells *in vitro* and have a higher GF content than whole blood supernatants (22). However, no correlation between specific GF concentrations and endothelial cell proliferation could be observed, suggesting that endothelial cell division is regulated by complex mechanisms which remain to be understood. The aim of this study was to determine the relative contribution of specific growth factors (VEGF, PDGF-BB, bFGF and EGF) released from PRPs on endothelial cell proliferation *in vitro*.

Material and methods

Blood harvesting and preparation of PRPs

Whole blood samples were collected from five donors who had provided their written informed consent. The study was approved by the Ethics Committee of Université Laval (Québec, QC, Canada; no. 2004-007). Samples of PRP were prepared with the platelet concentrate collection system (PCCS), according to manufacturer's instructions (3*i*; Implant Innovations Inc., West Palm Beach, FL, USA). Briefly, two PCCS bags per donor were filled with a mixture of 54 mL of whole blood and 6 mL of anticoagulant citrate dextrose (ACD) solution (Baxter HealthCare Corp., Deerfield, IL, USA) and the bags were centrifuged (International Equipment Company, Heedham Heights, MA, USA) at 850 *g* for 225 s. After removing the red blood cell fraction, the bags were centrifuged again at 850 *g* for 13 min and the platelet-poor plasma was removed from the PRP. Typically, 7 mL of PRP were obtained from each bag. The two samples of PRP from the same donor were pooled and incubated at room temperature for 2 h on a rotating platform to eliminate platelet aggregates and to homogenize the samples. Complete blood counts for whole blood samples and PRPs were performed on a Gen-S flow cytometer counter (Beckman-Coulter, Fullerton, CA, USA) by the Centre Hospitalier de l'Université Laval (Québec).

Activation of PRPs with calcium and thrombin

Bovine thrombin (Serologicals Proteins, Inc., Kankakee, IL, USA) and calcium chloride were added to activate PRP samples at the following final concentrations: 142.8 U/mL of thrombin and 14.3 mg/mL of $CaCl_2{\cdot}2H_2O$, as reported by Marx *et al.* (9). Calcium- and thrombin-treated PRP aliquots were incubated for 1 h at 37°C, since these conditions lead to the release of significant amounts of GFs in PRP supernatants (23,24). Samples were centrifuged for 10 min at 4000 *g*, and PRP supernatants were collected and immediately stored at −85°C until further analysis.

Determination of GF concentrations

Concentrations of GFs in PRP supernatants were determined by enzyme-linked immunosorbent assay (ELISA), as previously described (22,24). Concentrations of EGF were assayed using an ELISA Development kit according to the manufacturer's instructions (Peprotech Inc., Rocky Hill, NJ, USA). Levels of VEGF, PDGF-BB and bFGF were determined with DuoSet ELISA Development kits (R&D Systems, Inc., Minneapolis, MN, USA). Triplicate measurements were performed for all assays.

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex BioScience Walkersville, Inc. (Walkersville, MD, USA) and expanded in a complete culture medium consisting of M199 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Wisent, Saint-Laurent, QC, Canada), 15 U/mL of heparin, 100 U/mL of

penicillin, 100 μg/mL of streptomycin and 15 μg/mL of endothelial cell growth supplement (ECGS; Sigma Chemical Co., Oakville, ON, Canada). The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. Human umbilical vein endothelial cells from second and third passages were used in the experiments. The HUVECs were seeded at a density of 2500 cells per well in 0.25% gelatin-coated 96-well plates (Costar, Fisher Scientific, Ottawa, ON, Canada) in complete medium for 24 h. The medium was then removed and the cells were incubated for 3 days at 37°C in minimal medium (M199 medium with 100 U/mL of penicillin and 100 μg/mL of streptomycin) supplemented with untreated or treated PRP supernatants [10% (v/v)], as described below.

Inactivation of GFs by neutralizing antibodies

Vascular endothelial growth factor, PDGF-BB, EGF and bFGF (released in PRP supernatants and secreted by endothelial cells) were inactivated with specific monoclonal neutralizing antibodies (R&D Systems, Inc.) added to the minimal medium supplemented with 10% (v/v) PRP supernatant, and incubated with HUVECs for 3 days. The antibody concentrations necessary to achieve effective neutralization were determined by incubating HUVECs in minimal medium in the presence of a concentration of human recombinant GFs leading to maximal cell proliferation [25 ng/mL for VEGF; 5 ng/mL for PDGF-BB; 10 ng/mL for EGF (R&D Systems, Inc.) or 2 ng/mL for bFGF (Biosource International, Camarillo, CA, USA)] and various amounts of antibodies. It was shown that 1 ng/mL of anti-VEGF, anti-PDGF-BB and anti-EGF and 5 ng/mL of anti-bFGF produced the highest inhibition of endothelial cell proliferation (data not shown). These concentrations were then selected for the GF neutralization assays. Isotypic antibodies (R&D Systems, Inc.) were used as controls and showed an absence of non-specific cross-reactions of antibodies that could interfere with endothelial cell division. Experiments were performed in triplicate for each donor.

Removal of EGF by antibody-coated beads

Epidermal growth factor was physically removed from PRP supernatants, prior to their incubation with endothelial cells, in order to assess the mitogenic effect of EGF released in the supernatants. Briefly, 400 ng of protein G-coated Sepharose beads (Sigma Co.) were incubated for 20 min with 0.5 μg neutralizing antibodies directed against EGF (R&D Systems, Inc.). The optimal amount of antibodies to achieve maximal elimination of recombinant EGF was previously determined by ELISA testing (data not shown). The Sepharose beads, saturated with protein G-antibody complexes, were centrifuged at 350 *g* for 1 min, and the pellets were washed three times with 150 μL of phosphate-buffered saline in order to remove unbound antibodies. Antibody-saturated beads were incubated with PRP supernatants (250 ng beads/100 μL of supernatants) for 1 h at 4°C. After a 1 min centrifugation at 350 *g*, the supernatants, depleted of EGF, were added to endothelial cell cultures at 10% (v/v) final concentration, as described in the previous subsection.

Determination of HUVEC proliferation by [^{3}H]-thymidine incorporation

The effects of GF neutralization and EGF removal on HUVEC proliferation induced by PRP supernatants were determined by [^{3}H]-thymidine incorporation. After 2 days of incubation with PRP supernatants, [^{3}H]-thymidine (86.4 Ci/mmol specific activity; Perkin-Elmer, Boston, MA, USA) was added at a concentration of 0.2 μCi per well, and the cells were incubated for 16 h. The cells were washed twice with PBS, lysed with 100 μL of 1 N NaOH for 10 min, and the lysates were added to 5 mL of scintillation solution (Ecolite; ICN, Costa Mesa, CA, USA). [^{3}H]-Thymidine incorporation was assayed using a scintillation counter (LS-6500 Multi-Purpose Scintillation Counter; Beckman Coulter, Mississauga, ON, Canada). The proliferation rates were calculated by dividing the counts (in disintegrations per minute, d.p.m.) of a specific experiment by the counts (in d.p.m.) obtained in minimal medium supplemented with non-neutralized PRP supernatants. The effect of recombinant EGF (R&D Systems, Inc.) at 50, 100, 300 and 500 pg/mL on HUVEC proliferation was also tested.

Data analysis

To study the relationships between platelet counts and proliferation of endothelial cells, a univariate regression model was performed. Variables significant at $\alpha = 20\%$ were put into a multivariate regression model in which a stepwise approach was applied to select the most important variables. To study the effect of GF neutralization or removal, a randomized block design was applied using the patients as the block factor. In the case of EGF antibody neutralization, the treatment structure was a two-way factorial design with interaction: concentration (two levels) and growth factor. In the case of EGF removal, the treatment structure was only the growth factor. Following a significant effect, multiple comparisons were made using the Tukey adjustment method. Normality assumption was verified using the Shapiro–Wilk's statistic, and the homogeneity of variances was verified graphically using the residual plot. All the statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Whole blood from five healthy volunteers was collected, and PRPs were prepared with the PCCS system. Flow cytometry analyses revealed that the PCCS system allowed an increase in platelet concentration of 4.3-fold on average (range, 2.7- to 5.5-fold), with a platelet recovery rate of 63% (range, 44.1–75.0%), and elimination of about 98% of the red blood cells (Table 1). Monocyte and lymphocyte counts were increased by 2.0- and 4.3-fold, respectively, while neutrophil concentration in PRP remained similar to that found in whole blood.

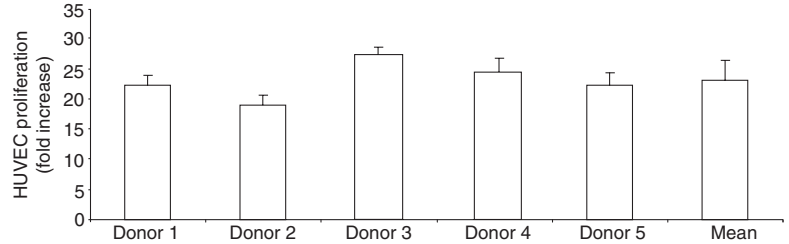
Table 1. Platelet, red blood cell and leukocyte counts in whole blood and PRPs from five donors

Cellular type	Whole blood (cells × 10^5/mL)	PRP (cells × 10^5/mL)	Yield (Folds)	Percentage recovery
Platelets	2100 ± 330	9100 ± 2900	4.3 ± 1.2	63 ± 11
Red blood cells	41000 ± 4300	6600 ± 2500	0.2 ± 0.1	2.3 ± 0.6
Neutrophils	28 ± 7	28 ± 10	1.0 ± 0.6	11.7 ± 9.2
Lymphocytes	16 ± 4	64 ± 13	4.3 ± 1.5	62 ± 17
Monocytes	4 ± 2	8 ± 5	2.0 ± 1.5	29 ± 21

All values are presented as means ± SD.

The concentrations of VEGF, PDGF-BB, bFGF and EGF released in supernatants from PRPs following activation with calcium and thrombin were determined for each donor (Fig. 1). On average, low levels of VEGF and bFGF were detected (mean, 189 and 39.5 pg/mL, respectively), while PDGF-BB and EGF were present at higher concentrations (mean, 27,190 and 513 pg/mL, respectively). Large differences in PDGF-BB and VEGF levels (11- and 7-fold variations, respectively) were observed between donors, in spite of small variation in platelet concentrations. As reported in Fig. 1, significant increases in endothelial cell proliferation (18- to 27-fold; $p < 0.0001$) were observed with the addition of 10% (v/v) PRP supernatants previously activated for 1 h with calcium and thrombin, as compared with the control culture (minimal medium).

In order to investigate the contribution of VEGF, PDGF-BB, bFGF and EGF, both released in PRP supernatants and produced by endothelial cells, in HUVEC proliferation, neutralizing antibodies, specific to each GF, were added to the medium during the proliferation assays. Neutralization of VEGF, PDGF-BB and bFGF had minor effects on cell proliferation (Fig. 2A–C). Interestingly, a significant inhibition of HUVEC proliferation was obtained following EGF neutralization in PRP supernatant from each donor, suggesting that EGF may be an important angiogenic factor in the experimental conditions used (mean reduction, 28%; range, 16–42%; $p < 0.0001$; Fig. 2D). In order to discriminate whether the angiogenic effects of EGF resulted from the EGF released from PRPs or from activated endothelial cells, an indirect technique of neutralization was employed. In this experiment, EGF released from PRPs was physically removed from the supernatant by using antibody-coated beads (mean EGF elimination, 54%), without eliminating the EGF synthesized by HUVECs. Even with partial elimination of the EGF released from PRPs, a significant reduction of HUVEC proliferation was observed (mean reduction, 40%; range, 30–59%; $p < 0.0001$; Fig. 3). These results suggest that EGF released in PRP supernatants (mean, 513 pg/mL) may represent an important activator of endothelial cell proliferation *in vitro*. The ability of recombinant EGF to induce HUVEC proliferation was then tested. As reported in Fig. 4, recombinant EGF increased HUVEC proliferation in a dose-dependent manner (at up to 300 pg/mL). More specifically, proliferation obtained at 100 pg/mL EGF was significant at $p = 0.03$.



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Mean
VEGF (pg/mL)	169 ± 11	39 ± 5	289 ± 24	275 ± 2	174 ± 12	189 ± 7
PDGF (pg/mL)	6470 ± 80	16900 ± 90	73250 ± 810	22230 ± 80	17080 ± 70	27190 ± 290
bFGF (pg/mL)	30.2 ± 0.3	39.6 ± 0.9	59.9 ± 1.9	43.8 ± 1.9	24.2 ± 0.5	39.5 ± 12.3
EGF (pg/mL)	490 ± 1	557 ± 5	649 ± 6	574 ± 11	295 ± 2	513 ± 4

Fig. 1. Effect of PRP supernatants on HUVEC proliferation. The HUVEC proliferation was determined by measuring [^{3}H]-thymidine incorporation after 2 days of treatment with 10% (v/v) PRP supernatants from five donors. All proliferation assays were performed in triplicate. Fold increase (means ± SD) was calculated by comparison with the control culture using minimal medium. Initial GF concentrations in PRP supernatant of each donor are indicated below each column.

Discussion

Growth factors are bioactive polypeptides that regulate the proliferation and differentiation of cells from epithelium, bone and connective tissues. It has been proposed that platelets constitute a reservoir of various GFs, which, once released, may positively regulate the healing of oral surgery and/or periodontal wounds (9). In this study, PRPs were prepared with the PCCS system from five healthy donors. Platelet yields (4.3-fold increase) and percentages of recoveries (63%) were similar to previous reports using this system (22–25).

The concentrations of VEGF, PDGF-BB, bFGF and EGF in supernatants collected after activation of PRPs with calcium and thrombin were determined for each donor. On average, PDGF-BB was present in high concentrations (27,190 pg/mL), while moderate amounts of VEGF and EGF were detected (189 and 513 pg/mL, respectively) and trace amounts of bFGF (39.5 pg/mL) were found in supernatants from activated PRPs. These results are consistent with the concentrations previously reported in such preparations (23,24,26,27). Important variations in GF concentrations were observed between individuals, which may explain, in part, some of the conflicting results obtained with PRPs in different clinical studies (1,17,22,23,26).

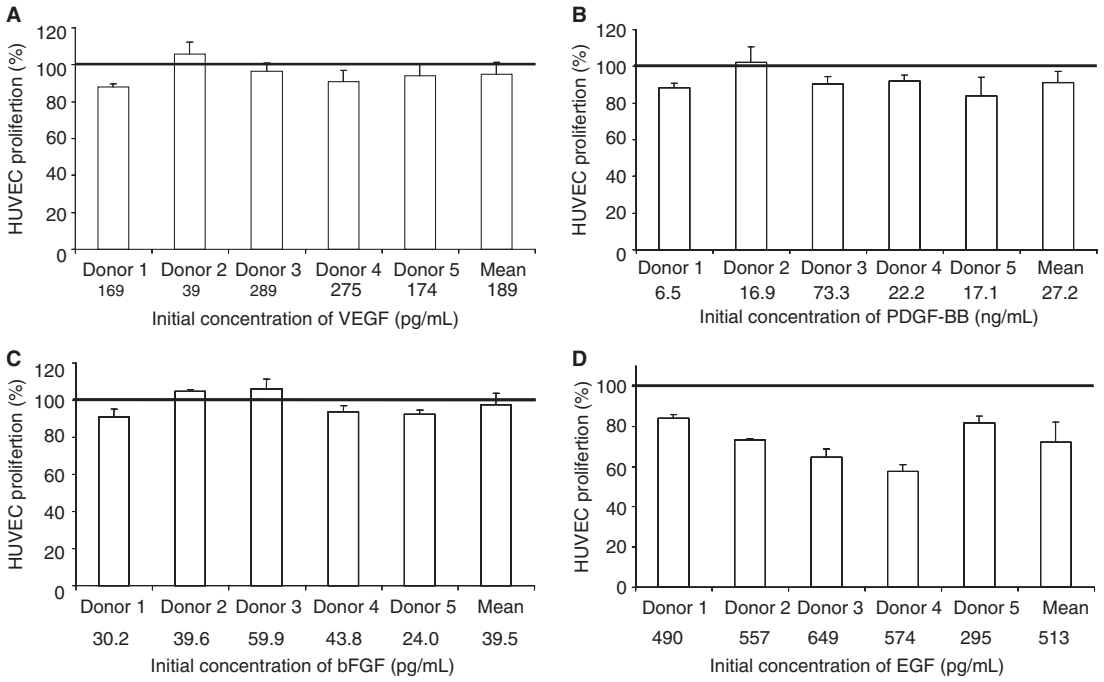


Fig. 2. Effect of GF neutralizing antibodies on PRP supernatant-induced HUVEC proliferation. All neutralization assays were performed in triplicate for each donor and were compared with non-treated supernatants, to which 100% proliferation was assigned. (A) Neutralization with 1 ng/mL of anti-VEGF; (B) neutralization with 1 ng/mL of anti-PDGF-BB; (C) neutralization with 5 ng/mL of anti-bFGF; and (D) neutralization with 1 ng/mL of anti-EGF. Initial GF concentrations in PRP supernatants for each donor prior to neutralization are indicated below each column.

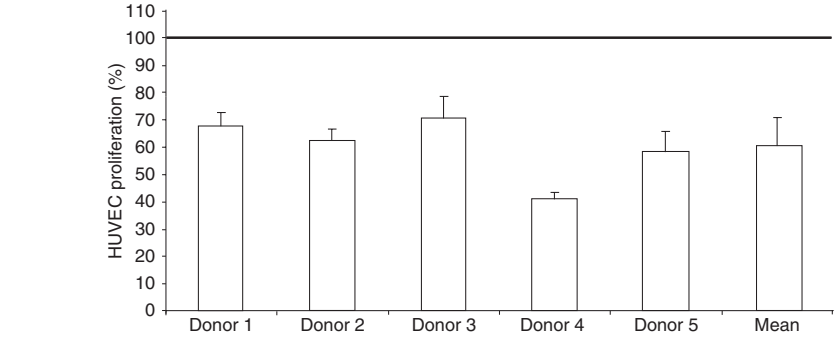


Fig. 3. Effect of EGF removal from of PRP supernatants on HUVEC proliferation. The assay using EGF-depleted PRP supernatants was performed in triplicate and compared with non-treated supernatants, to which 100% proliferation was assigned. Initial concentrations and residual concentrations in PRP supernatants after neutralization and percentages of elimination are indicated below the columns for each donor.

Human umbilical vein endothelial cells were incubated with PRP supernatants, in order to assess the mitogenic potential of the released GFs. Endothelial cell proliferation, which represents a key step in the angiogenic process, was chosen as an *in vitro* model, based on the work of Knighton *et al.* (28), who demonstrated that the use of thrombin-treated platelets significantly increased the healing rate of the rabbit cornea, which directly correlated with the induction of angiogenesis. In this study, we found that supernatants collected after PRP activation with thrombin and calcium significantly enhanced proliferation of HUVECs *in vitro*, which is in agreement with our previous reports (15,22–24).

In order to better understand the roles of specific GFs released from PRPs on the angiogenic process, HUVECs were incubated with PRP supernatants in which specific GFs released from PRPs were neutralized with antibodies specific to VEGF, PDGF-BB, bFGF or EGF. Neutralization of VEGF and bFGF, which are known to be potent stimulators of endothelial cell proliferation (29,30), did not affect HUVEC proliferation. This may be explained by the limited amounts of VEGF and bFGF initially present in PRP supernatants (mean, 189 and 39.5 pg/mL, respectively) and by the limited production of these GFs by endothelial cells. This is supported by Ferrara *et al.* (31), who have indicated that endothelial cell proliferation with VEGF is maximal at 1–4 ng/mL concentrations. We have previously reported that bFGF is more concentrated in PRP supernatants than in whole blood supernatants (3-fold

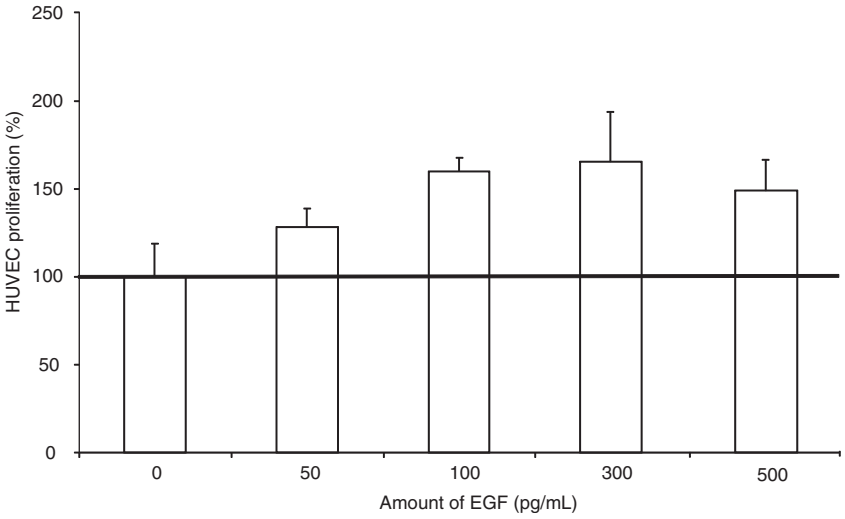


Fig. 4. Effect of recombinant EGF on HUVEC proliferation. The HUVEC proliferation was determined by measuring [^{3}H]-thymidine incorporation after 2 days of treatment with recombinant EGF at 50, 100, 300 and 500 pg/mL. All proliferation assays were performed in triplicate. A value of 100% proliferation was assigned to the control culture using minimal medium.

higher; 22); however, the amount found in PRP supernatants may still not be high enough to significantly modulate endothelial cell division.

Platelet-derived growth factor BB is a chemotactic factor for monocytes, neutrophils, fibroblasts, mesenchymal stem cells and osteoblasts, and is released in high quantities at injured sites (27,32–34). In the present study, high concentrations of PDGF-BB were also detected in PRP supernatants (mean, 27,190 pg/mL). However, PDGF-BB neutralization did not significantly affect endothelial cell proliferation, which supports the notion that endothelial cells do not have receptors for PDGF-BB (35). The absence of mitogenic effects of this GF on HUVEC proliferation was confirmed by dose–response experiments, in which incremental concentrations of recombinant PDGF-BB did not stimulate HUVEC proliferation (data not shown).

Lastly, neutralization of EGF in PRP supernatants by the use of specific antibodies caused a significant decrease in endothelial cell proliferation, suggesting that this GF released from activated PRPs may play a key role in endothelial cell division. Since endothelial cells can also synthesize GFs, such as EGF (36), treatment of PRP supernatants with anti-EGF antibody-coated beads was performed. With these EGF-free PRPs, HUVEC proliferation was relatively similar to that observed in the presence of neutralizing antibodies to EGF, suggesting that the amount of EGF synthesized by endothelial cells is somewhat limited.

While Mehta & Besner (37) have reported that EGF is able to induce endothelial cell migration and to promote endothelial tube formation, these authors found that EGF was unable to promote endothelial cell division at concentrations between 0.1 and 20 ng/mL. This result differs from our findings, in which a 1.6-fold significant increase in HUVEC proliferation was observed in the presence of 100 pg/mL EGF. Recently, in agreement with our study, Shao *et al.* (38) demonstrated that EGF can promote proliferation of human corneal endothelial cells in a dose-dependent fashion.

In conclusion, PRP supernatants are highly mitogenic for endothelial cells *in vitro*, and in the conditions tested, EGF released in PRP supernatants may represent an important activator of endothelial cell division *in vitro*, which may explain in part the endothelial cell proliferation related to PRP supernatants.

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