Platelet gel-released supernatant modulates the angiogenic capability of human endothelial cells

Anna Rughetti[1], Ilaria Giusti[2], Sandra D'Ascenzo[2], Pietro Leocata[2], Gaspare Carta[3], Antonio Pavan[4], Luigi Dell'Orso[1], Vincenza Dolo[2]

[1] Servizio di Medicina Trasfusionale, Ospedale "San Salvatore", L'Aquila; [2] Dipartimento di Medicina Sperimentale, Università di L'Aquila, L'Aquila; [3] Dipartimento di Scienze Chirurgiche Università di L'Aquila, L'Aquila; [4] Dipartimento di Medicina Sperimentale, Università "La Sapienza", Roma, Italy.

Background. Platelet gel is used to facilitate wound healing in virtue of the growth factors released from activated platelets at the site of lesion, but little is known about the specific mechanisms underlying cellular repair.

Aims. To evaluate, *in vitro,* cellular effects of different concentrations of platelet gel -released supernatant on endothelial cells.

Material and methods. Platelet concentrate was produced at the Service of Immunohaematology and Transfusion of *San Salvatore* Hospital of L'Aquila, using multiple bags. Platelet gel was obtained by adding thrombin and calcium gluconate to the concentrates and then centrifuging to recover the supernatant.

Human umbilical vein endothelial cells were isolated from umbilical cord veins and grown in appropriate conditions. To study their viability, cells were treated with different concentrations of supernatant and XTT assays were performed on the 3 days following treatment. Endothelial cell motility and invasiveness were assayed using modified Boyden chambers with filters coated with 0.1% gelatin (for the motility test) or with a thick layer of the reconstituted basement membrane Matrigel (for the invasion test). The supernatant, added at various concentrations to the lower compartment of the chamber, was used as an attractant. Umbilical cells were added to the upper compartment of the chamber. After 4 hours (for the motility test) or 6 hours (for the invasion test), filters were stained and the migrated cells in five high-power fields were counted.

Results. When used at specific concentrations, platelet gel-released supernatant is able to induce proliferation and to stimulate motility and invasiveness of endothelial human cells. Higher concentrations induce a reversion of the stimulatory processes.

Conclusions. There is a large body of evidence indicating that platelets and their derivatives have the potential for a substantial therapeutic role in tissue regeneration. The results of this *in vitro* study highlight the need for an in-depth analysis of technical protocols for the most appropriate and effective use of platelet gel for *in vivo* applications.

Key words*:* platelet gel, endothelial cells, wound repair, motility, invasion.

Introduction

In recent years the interest in blood components, predominantly autologous ones, as topical applications to stimulate tissue growth, has rapidly widened to various clinical uses and specialist fields because of the possible therapeutic effects of blood cells and/or plasma-derived factors in various pathological states [1].

Platelet gel is a blood component for topical use, obtained from platelet concentrates activated with calcium and thrombin: following activation, the platelets in the gel release a series of biologically active molecules (predominantly growth factors), which diffuse into the surrounding environment, triggering and enhancing wound healing[2,3]. Indeed, the growth factors released by platelets accelerate the proliferation of chondrocytes, osteoblasts, fibroblasts and endothelial cells[4-6]. In the last few years, there has been a considerable increase in the use of platelet gel to improve bone regeneration and wound healing in many fields such as orthopaedics, maxillofacial surgery, dentistry, plastic surgery[7] and the repair of skin ulcers[8].

Despite this, platelet gel continues to be used in a completely empirical way, based mainly on the experience of individual doctors. It is, therefore, necessary to obtain experimental *in vitro* data which could be determine the most appropriate and effective use of platelet gel for *in vivo* clinical applications.

Materials and methods
Preparation of the supernatant released by the platelet gel

The platelet gel was prepared from the buffy coats of standard homologous blood donations; from each of eight male donors, 450 mL of whole blood were collected into triple bags (Teruflex triple bags with CPD/SAGM, Terumo, Italy, Rome). As required by law (Legal Decree of March 3, 2005 and Law n. 219 of October 21, 2005), all the donors signed informed consent.

The whole blood was fractionated by a first centrifugation of the bag for 10 minutes at 22 °C in a Heraeus Cryofuge 6000i centrifuge (AHSI Spa, Massa Martana, PG, Italy) RCF 462 (1,200 rpm) in order to obtain platelet-rich plasma (PRP) and red blood cell concentrate (RCC); the PRP thus obtained was subsequently centrifuged a second time for 6 minutes at 22 °C, RCF 3932 (3,500 rpm) in order to produce a platelet concentrate and platelet-poor plasma (PPP). Finally, the platelets were hyperconcentrated in 10-15 mL of plasma.

The platelet gel was produced by placing aliquots of platelet concentrate in Vacutainer Plus test-tubes containing 5 NIH Units of thrombin (367817, Becton Dickinson, Plymouth, UK), adding calcium gluconate (Bioindustria Laboratorio Italiano Medicinali S.p.A.,

Novi Ligure, AL, Italy) 1:20 and leaving the mixture to clot for 5 minutes at 37 °C. The coagulum was then centrifuged for 10 minutes at 1,200 rpm in order to obtain a supernatant rich in growth factors released by the activated platelets. The supernatant was subjected to a series of centrifugations (10 minutes each, at 1,200-4,000 rpm) to separate the red blood cells, debris and stroma and then used fresh for the tests of cell proliferation, motility and invasion.

The initial concentration of platelets in the PRP differed between the preparations ($3x10^6$-$7.5x10^6$ plt/mL); in order to obtain the final platelet concentrations, the supernatant released by the platelet gel was diluted with appropriate amounts of Dulbecco's Modified Eagle's Medium (DMEM) (for the motility and invasion tests) or DMEM + 2.5% foetal calf serum, FCS (for the proliferation test).

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins and grown in an incubator at 37 °C in 5% CO_2, in flasks lined with gelatin 1% in DMEM supplemented with 10% NCS, 10% FCS, 20 mM HydroxyEthil Piperazine Ethanol Sulfonic (HEPES), 6 U/mL heparin, 2 mM glutamine, 50 µg/mL endothelial cell growth factor (crude extract from bovine brain), penicillin and streptomycin. The cells were used between the third and fourth passage.

Cell proliferation tests

Cell proliferation was evaluated using the XTT method (Sigma, St. Louis, MO, USA). The metabolic reduction of XTT by living cells produces a coloured, non-toxic formazan that is soluble in water; the amount of this substance (which can be measured by an ELISA reader) is directly proportional to the number of viable cells[9].

HUVEC were seeded (1,500 per well) into 96-well plates. The cells were made to adhere in complete medium for 24 hours; subsequently, various concentrations (0-$7x10^6$ plt/mL) of the supernatant released from the platelet gel were added to the medium. HUVEC treated with complete medium were used as a positive control, whereas the negative control consisted of HUVEC grown in DMEM supplemented with 2.5% FCS. The cells thus treated were incubated at 37°C in 5% CO_2. Cell proliferation was evaluated after 24, 48 and 72 hours using the XTT assay. After

4 hours of incubation with XTT, the optical density was read at 450 nm.

Every experiment was conducted in triplicate and repeated at least twice. The data are expressed as the mean ± standard deviation.

Motility and invasion tests

The migration assay enables an *in vitro* evaluation of the capacity of cells to move towards a soluble chemoattractant. The invasion assay measures, *in vitro,* the invasive capacity of cells, because the cells must pass through a layer of Matrigel, a compound that mimics the composition of the extracellular matrix.

The motility and invasiveness of the endothelial cells were evaluated in a Boyden chamber using PVP-free polycarbonate filters (Nucleopore) with a pore diameter of 8 μm. The chemoattractant stimulus was the supernatant, added to the lower chamber at various concentrations (0-5x10^6 plt/mL). FCS-free medium and supernatant from NIH-3T3 were used as negative and positive controls, respectively.

For the chemotaxis test (i.e. the migration test) the filters were covered with gelatin 0.1%, whereas for the invasion test, the filters were covered with a thin layer of Matrigel 0.5 mg/mL reconstituted basal membrane (Beckton Dickinson, Bedford, MA, USA).

The HUVEC were detached, washed in DMEM-0.1% bovine serum albumine, resuspended in the same medium at a concentration of 5x10^5/mL and added to the upper compartment of the chamber. After 4 hours (for the migration test) or 6 hours (for the invasion test) of incubation at 37 °C, the filters were stained with Diff-Quik (Baxter, Dudingen, Switzerland) or crystal-violet 1% in methanol and the cells that had migrated were counted in five random fields at high-power magnification.

Every experiment was carried out in triplicate and repeated at least twice. The data are expressed as the mean ± standard deviation.

Results
The supernatant released by platelet gel induces proliferation of endothelial cells

In order to evaluate the capacity of the platelet gel-released supernatant to stimulate proliferation of endothelial cells, HUVEC were incubated (for 24, 48 and 72 hours) with the supernatant recovered after centrifuging the platelet gel and diluting it

to obtain different concentrations ranging from 0 to 7x10^6 plt/mL (300,000 plt/mL, 500,000 plt/mL, 750,000 plt/mL, 1.25x10^6 plt/mL, 1.75x10^6 plt/mL, 2.25x10^6 plt/mL, 2.75x10^6 plt/mL, 3.25x10^6 plt/mL, 4x10^6 plt/mL, 5x10^6 plt/mL, and 7x10^6 plt/mL).

As shown in figure 1, the supernatant was able to induce cell proliferation, compared to that of untreated cells, already at a minimum concentration of 300,000 plt/mL. The maximum proliferation after 24 and 48 hours was obtained starting with a concentration of 1.75x10^6 plt/mL (producing 2.65- and 3-fold increases in proliferation compared to the untreated cells, respectively); higher concentrations did not stimulate proliferation further, since a plateau was reached. Indeed, at the highest concentrations, there was a tendency to an inversion in the effect on proliferation, with values returning to similar to those of untreated cells.

The effect of stimulation was much more marked after treatment for 72 hours: the increase in proliferation reached a maximum (about 6-fold higher than that of untreated cells) using a concentration of about 1.25x10^6 plt/mL. Concentrations above this value inhibited proliferation; at the highest concentration used (7x10^6 plt/mL), proliferation returned to being comparable to that occurring in untreated HUVEC.

Effect of the supernatant released by platelet gel on cell invasion and motility

In order to determine whether the supernatant released by the platelet gel is able to stimulated angiogenesis, the supernatant's capacity to stimulate two important angiogenic functions of endothelial cells, motility and invasiveness, was evaluated.

In order to do this, the platelet gel-released supernatant was used as the chemoattractant in migration and invasion tests. These tests demonstrated that the supernatant was able to stimulate both processes in a dose-dependent manner (Figure 2, A and B); the greatest stimulation was obtained, for both processes, using a concentration of 1.5x10^6 plt/mL. At this concentration, there was an approximately 3-fold increase in motility (Figura 2 A), compared to that of unstimulated cells; higher concentrations also stimulated motility but to a lesser degree (about double that of the control cells). The results of the invasion test (Figure 2 B) paralleled those of the migration test: the maximum stimulation occurred at a concentration
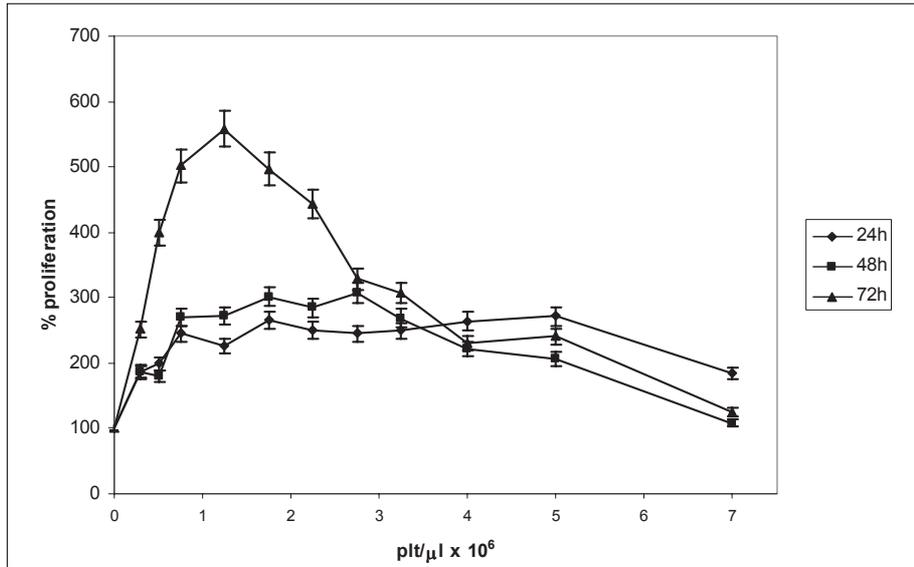
Figure 1 - HUVEC proliferation. The supernatant obtained after platelet gel centrifugation stimulates endothelial cell proliferation with a dose-dependent manner. Data (Mean ± SD of triplicates) are the % of proliferation; proliferation of non treated HUVEC was set as 100%.
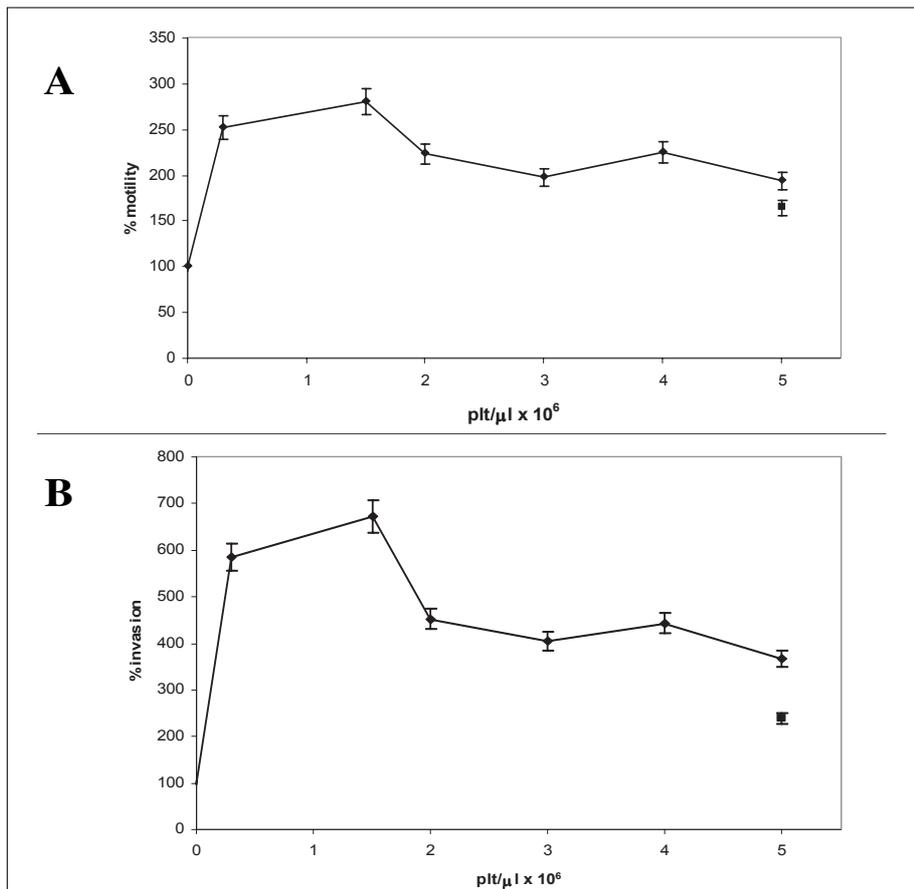

Figure 2 - HUVEC motility (A) and invasion (B) tested in Boyden chamber using supernatant as the attractant. NIH-3T3 (squares) was used as a reference stimulus. Data (Mean ± SD of triplicates) are the % of cells migrated; motility and invasion of non treated HUVEC were set as 100%.

of 1.5×10^6plt/mL (the migration of treated cells being about 6.5-fold greater than that of untreated HUVEC) and the effect diminished at higher concentrations (about a 4-fold incease in migration).

Discussion and conclusions

Wound healing is a complex process that involves various different types of cells, growth factors and other proteins that interact with each other leading to fast, efficient repair of the lesion. Platelets play a primary role in tissue repair/regeneration not only because of their haemostatic properties, but also because of their capacity to release a series of growth factors involved in repair of the lesion[3].

For this reason, the use of blood components, predominantly autologous, for purposes other than the classical ones of transfusion support, has rapidly expanded in the last few years to various clinical applications and specialist fields: orthopaedics, maxillofacial surgery, dentistry, plastic surgery[7] and repair of cutaneous ulcers[8]. Of the various blood components available for topical use, platelet gel has attracted particular attention, because it is the source of growth factors capable of stimulating tissue regeneration/ repair.

In fact, given its high concentration of platelets, platelet gel contains a notable amount of growth factors secreted by the activated platelets. These growth factors include platelet-derived growth factor, tumour growth factor-β, vascular endothelial growth factor, epithelial growth factor[10], insulin-like growth factor-I and basic fibroblast growth factor [11].

Platelet gel facilitates wound healing through degranulation of the platelet α granules that contain the growth factors.

These factors, once secreted, bind rapidly to their respective receptors; various studies have shown that osteoblasts, fibroblasts, epidermal cells and endothelial cells express receptors for the growth factors contained in platelet granules[12]. The binding of the growth factors to their receptors activates cells in the surrounding tissues such that these repair the lesion[13].

Over the years, many studies have been conducted to elucidate the mechanisms involved in the tissue repair induced by platelet concentrates and it has been found that platelet gel is able to induce proliferation of fibroblasts[1], endothelial cells[6,14] and osteoblasts[7]. Despite this, it is still not completely clear which

parameters should be used in clinical practice to obtain the best healing of lesions. For this reason, we focused on understanding the relationship between the concentration of the supernatant released by the platelet gel and changes in cellular parameters of human endothelial cells. In fact, during the repair of a wound, endothelial cells are involved in the formation of new blood vessels from the intact vessels present in the margins of the lesion[7].

The current study confirms that some platelet-released factors can, *in vitro*, stimulate proliferation of endothelial cells and also the migratory and invasive capacity of these cells. It is, however, clear that different concentrations of the factors do not have the same efficacy in inducing these processes: excessively high concentrations have an inhibitory effect on the processes. The most effective concentration for stimulating proliferation was that obtained from diluting the platelet gel-released supernatant to a concentration of about 1.5×10^6plt/mL; lower or higher concentrations had less effect. The same concentration was most efficient in stimulating the processes of migration and invasion: although all the tested concentrations of the supernatant stimulated these processes, the maximum stimulation occurred, as for the previous tests, when the concentration was 1.5×10^6 plt/mL.

This study contributes to understanding the role of platelet gel and its exudate in the healing of wounds, demonstrating certain critical parameters of the preparation: our data suggest that too high concentrations of platelets and, therefore, probably of growth-stimulating factors, can have counterproductive effects on wound healing.

Further studies are necessary to reach a better understanding of the complex biological processes triggered by these factors *in vivo*. The utility of future *in vitro* studies will be to provide parameters of cell function useful for defining guidelines on the clinical applications of platelet gel.

Authorship

Anna Rughetti and Ilaria Giusti contributed equally to this work.

References

1) D'Agostino E, Fratellanza G, Caloprisco G, et al. Effetto del gel piastrinico sulla proliferazione cellulare *in vitro*. La Trasf del Sangue 2002; **47**: 429-33.

2) Borzini P, Mazzucco L. Platelet gels and releasates. Curr Opin Hematol 2005; **12**: 473-79.

3) Carlson NE, Roach RB. Platelet-rich plasma. Clinical applications in dentistry. JADA 2002; **133**:1383-86.

4) Okuda K, Kawase T, Momose M, et al. Platelet-rich plasma contains high levels of platelet-derived growth factors and transforming growth factor-beta and modulates the proliferation of periodontally related cells in vitro. J Periodontol 2003; **74**: 849-57.

5) Mazzucco L, Cattana E, Orecchia S, et al. Enhancing of fibroblast proliferation by platelet extract. Vox Sang 2004; **87** (Suppl S2-S16):13.

6) Pintucci G, Froum S, Pinnell J, et al. 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). Thromb Haemost 2002; **88**: 834-42.

7) Fréchette JP, Martineau I, Gagnon G. Platelet-rich plasmas: growth factor content and roles in wound healing. J Dent Res 2005; **84**: 434-9.

8) Mazzucco L, Medici D, Serra M, et al. The use of autologous platelet gel to treat difficult-to-heal wounds: a pilot study. Transfusion 2004; **44**:1013-8.

9) Roehm NW, Rodgers GH, Hatfield SM, et al. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 1991; **142**: 257-65.

10) Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg 2004; **62**: 489-96.

11) Borzini P, Mazzucco L. Tissue regeneration and in loco administration of platelet derivatives: clinical outcome, heterogeneous products, and heterogeneity of the effector mechanisms. Transfusion 2005; **45**: 1759-67.

12) Marx RE, Carlson ER, Eichstaedt R, et al. Platelet rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998; **85**: 638-46.

13) Nishimoto S, Oyama T, Matsuda K. Simultaneous concentration of platelets and marrow cells: a simple and useful technique to obtain source cells and growth factors for regenerative medicine. Wound Rep Reg 2007; **15**: 156-62.

14) Martineau I, Lacoste E, Gagnon G. Effects of calcium and thrombin on growth factor release from platelet concentrates: kinetics and regulation of endothelial cell proliferation. Biomaterials 2004; **25**: 4489-502.

Correspondence: Prof. Vincenza Dolo,
Dipartimento di Medicina Sperimentale,
Università di L'Aquila,
Via Vetoio-Coppito 2,
I-67100, L'Aquila - Italy
e-mail: dolo@univaq