Platelet gel stimulates proliferation of human dermal fibroblasts in vitro

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ABSTRACT

Background: Platelet gel is a source of many soluble proteins that can modulate tissue regeneration. Dermal fibroblasts play an important role in tissue remodeling and wound healing.

Objective: The study was performed to investigate the influence of platelet gel and its active ingredients on the proliferation of human dermal fibroblasts in vitro.

Methods: A fibroblast culture was established from a normal human skin biopsy. Platelet gel was prepared from platelet-rich plasma (PRP) following the addition of calcified thrombin solution. Cell proliferation was measured by MTT assay.

Results: We showed that platelet gel, PRP, and thrombin stimulate the proliferation of dermal fibroblasts, and that stimulation by PRP is dose dependent.

Conclusion: Platelet gel can be used to treat chronic skin defects.

KEY WORDS

platelet gel, platelet-rich plasma, dermal-fibroblasts, proliferation, MTT assay

Tissue repair normally begins with clot formation and platelet degranulation, leading to the release of various cytokines and coagulation factors, which modulate inflammatory response. To date, more than 30 different cytokines have been found in platelets; PDGFs, TGFs, EGF, and IGF are among those most studied (1). Platelet gel is produced by mixing two solutions: platelet-rich plasma and calcified thrombin solution. The PRP can be prepared by aphaeresis or can be separated from fresh anticoagulated blood by simple centrifugation, which concentrates platelets up to six times the baseline count in whole blood (2). The production process itself (2), as well as the addition of calcified thrombin, activates platelets, which in turn release cytokines. At the same time, fibrinogen converts into a fibrin clot. Platelets attach to the fibrin web forming the platelet gel, which can be applied directly to injured tissue to enhance its regeneration. There are several commercially available devices that simplify the production of platelet-rich plasma from a patient's blood (Vivostat® [Vivolution A/S], SmartPReP® [Harvest Technologies], and Fibrinet® [Cascade Medical Enterprises]). Platelet gel is being used in a variety of clinical procedures: as a tissue sealant (3), as a skin healing enhancer (4), or for dental and bone regeneration (5–7). Fibrin sealant, a well-known hemostatic

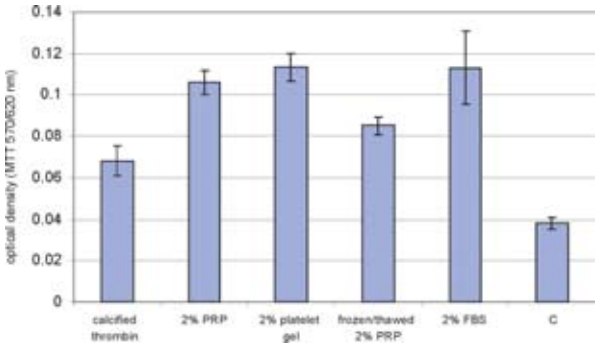

Figure 1. Proliferation of fibroblasts in the presence of various components of the platelet gel (MTT assay). Concentration of platelets in PRP was 887 x 10^9/L; calcified thrombin (10 IU/mL); 2% FBS in the medium as a positive control; medium without any additives as a negative control (C). Results shown are the means of four parallel cultures ± SD.

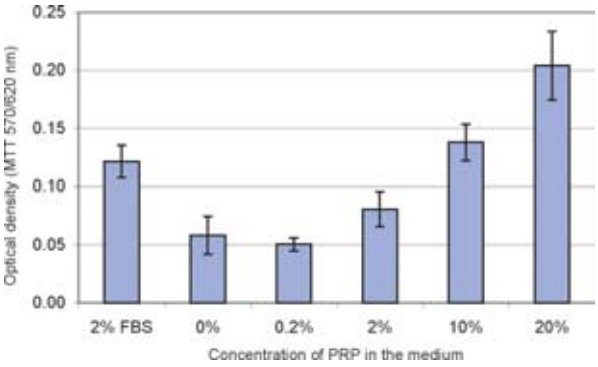

Figure 2. Proliferation of fibroblasts in the presence of platelet-rich plasma (PRP). Concentration of platelets in PRP was 1416 x 10^9/L; 2% FBS as a positive control. Results shown are the means of four parallel cultures ± SD.

material, is also a blood-derived product that contains proteins for blood coagulation but lacks platelets and cytokines. In addition, it promotes wound healing and skin engraftment, and serves as a scaffold for fibrin-based cultured skin substitutes (8–10).

Dermal fibroblasts are one type of essential cell in

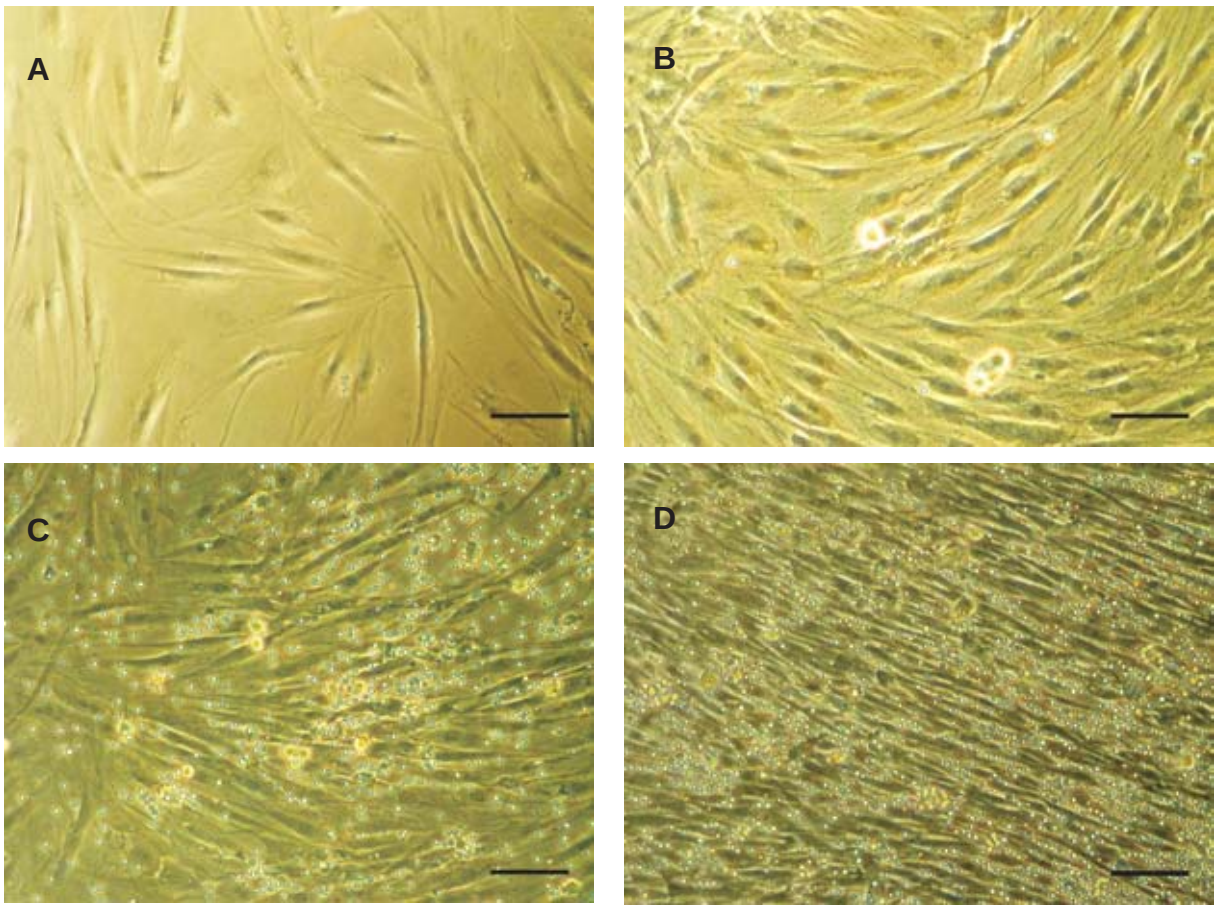

Figure 3. Fibroblast cultures after 4 days of incubation with 0% PRP (A), 2% FBS (B), 2% PRP (C) and 20% PRP (D). PRP contained 1416 × 10^9 platelets/L. The scale bars are 50 μm.

the dermis. They play an important role in repairing skin wounds by providing growth factors and extracellular matrix proteins that attract new cells, which eventually restore the physiological continuity of the skin. When the balance between cytokine and extracellular matrix protein synthesis and degradation shifts towards degradation, skin cells are no longer able to completely restore normal skin, and the damaged tissue progresses into a chronic wound. A more advanced approach to healing chronic wounds is the application of cytokines into the poorly healing tissue, so that balance is brought back to the synthesis and cell proliferation (11, 12). Platelet gel, as a source of various cytokines, could be an option to enhance the regeneration process.

The aim of this study was to investigate the influence of platelet gel and its compounds on the proliferation of human dermal fibroblasts in a cell culture.

Materials and methods

Fibroblast culture

A normal adult human skin sample was obtained from reductive surgery. The skin was cut into small pieces, which were incubated overnight at 37 °C in a collagenase solution (200 U/ml) (GibcoBRL, Invitrogen). Subsequently, the epidermis was discarded and dermal cells were isolated from the dermal layer of the skin after another overnight digestion with collagenase. Dermal cells were subcultured in a complete fibroblast medium supplemented with insulin, bFGF, 2% FBS, gentamicin (50 µg/ml), and amphotericin B (2 µg/ml) (FGM-2, Cambrex) at 37 °C in a humidified CO_2 incubator.

Platelet gel preparation

Platelet-rich plasma was prepared from venous blood obtained from healthy volunteers after obtaining their signed informed consent. Blood samples were collected in 9 mL tubes with tri-sodium citrate anticoagulant. The blood was first separated into two layers by centrifugation at 272 g for 7 min. The upper layer was collected and centrifuged at 1,288 g for 7 min. PRP was collected from the resulting 1–1.5 mL sediment, and the platelets were counted (Cell-DYN 3200, Abbott). The platelet gel was prepared by mixing equal parts of PRP and calcified thrombin 500 IU/ml in 40mM solution (Tissucol, Baxter-Hyland Immuno). The study was approved by the National Medical Ethics Committee.

Fibroblast proliferation assay

Fibroblasts were seeded in 24-well plates in 0.5 mL of the FGM-2 medium with 2% FBS. When cells reached 30% of confluent growth, the medium was washed off and cells were incubated for 24 hours in FGM-2 without serum. The medium was refreshed before adding the platelet gel compounds. Six different growth conditions were tested in the FGM-2 medium without FBS: calcified thrombin (10 µl), PRP (10 µl), platelet gel (20 µl), frozen/thawed PRP (10 µl), FGM-2 medium (negative control), and 2% FBS (positive control). The effect of different concentrations of PRP on cell proliferation was also studied. After 4 to 5 days of incubation, the proliferation of fibroblasts was measured with an MTT assay (Sigma-Aldrich Chemie). This is a colorimetric assay that measures the chemical reduction of MTT (3-[4.5-Dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) into formazan, which is directly proportional to the number of viable cells in the tested culture. Cultures were incubated 2 to 3 hours in a culture medium with 0.5 mg/mL of MTT. The resulting formazan was then eluted with acidified isopropanol (0.04 N HCl in isopropanol) and the optical density was measured at 570 nm, then subtracted for the optical density at 620 nm. To obtain the optical densities of all tested media, another 24-well plate was prepared as described above, but the fibroblasts were excluded. At the end of the assay, these values were subtracted from the experimental optical densities of cell culture supernatants. Proliferation was measured in four replicates. Optical densities were expressed as the mean ± standard deviation. Differences between means were assessed by Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant. Experiments were performed four times with PRP obtained from four different donors.

Results

The platelet-rich plasma prepared by a simple centrifugation process from whole blood resulted in a concentration of platelets approximately three to four times the baseline count in whole blood. All components of the platelet gel significantly stimulated the growth of fibroblasts when compared to the negative control (Figure 1; $p < 0.0001$). The calcified thrombin alone also showed a slightly positive effect on fibroblast growth, although the stimulation was not as strong as for PRP, frozen/thawed PRP, or platelet gel. Five different concentrations of PRP (0, 0.2, 2, 10, and 20%) were tested to determine the maximal stimulating effect on fibroblast proliferation. Fibroblast growth was enhanced in a dose dependent manner. 20% PRP showed the strongest effect, whereas 0.2% PRP did not affect growth at all (Figure 2). All fibroblast cultures retained normal morphology. After only 4 days of incubation in the presence of 20% PRP, fibroblasts completely covered the surface of the wells, forming a dense layer of cells (Figure 3). Unfortunately we could not test higher concentrations in this experiment because all the PRP was al-

ready used. In a further experiment (data not shown), we were able to test higher concentrations of PRP, including 100% PRP, but we could not measure the control media with MTT because of the lack of PRP. Nevertheless, after 4 days of incubation we observed the highest proliferation in 100% PRP with light microscopy.

Discussion

Platelet gel is a biological source of various cytokines (13), which stimulate the proliferation of osteoblasts, gingival fibroblasts (14), mouse embryonic fibroblasts (15), stromal stem cells (16), and endothelial cells (17), and enhance healing of skin ulcers (4, 18, 19). In our study we showed that allogeneic platelet gel stimulates the proliferation of primary dermal fibroblasts in vitro. The MTT assay, which we used to measure the proliferation, is based on the reduction of MTT catalyzed mainly by mitochondrial enzymes (but also by a number of other non-mitochondrial enzymes), which are abundantly present in fibroblasts as well as in platelets (20, 21). This fact was taken into consideration by subtracting the optical densities (PRP only, without cells) from those assessed in fibroblasts containing cultures incubated with PRP. Cells were cultured in a serum-free medium to avoid the stimulative effect of FBS. We showed that at least 2% PRP (approx. 28 x 10^6 platelets/mL) stimulates proliferation and that the stimulation is dose dependent, which is consistent with findings reported by other studies (22, 16). We did not observe any cytotoxic effects of PRP on proliferation and morphology. Frozen PRP and calcified thrombin were also able to stimulate cell proliferation. Frozen/thawed PRP retained its capacity to form a clot when it was mixed with calcified thrombin. This finding could be useful in preparing PRP in advance and for storage in a freezer for multiple applications (13). It has been shown before that thrombin acts as a potent fibroblast mitogen. Experiments were conducted examining the in vitro dose dependent effects of thrombin on proliferation of human skin fibroblasts in a fibrin-sealing system (23, 24). The role of growth factors promoting tissue regeneration has been well studied (25–28). A recombinant human PDGF-BB (becaplermin) has been developed and is commercially available as Regranex®. It is being used successfully to treat chronic wounds (29, 30) and periodontal defects (31). Our study contributes to this kind of knowledge because it shows the positive effect of platelet gel on the growth of human dermal fibroblasts in vitro.

Abbrevetation

PRP- platelet-rich plasma
MTT - (3-[4.5-Dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) FBS - fetal bovine serum

REFERENCES

1. Weibric G, Buch RS, Kleis WK, Hafner G, Hitzler WE, Wagner W. Quantification of thrombocyte growth factors in platelet concentrates produced by discontinuous cell separation. Growth Factors 2002; 20(2):93–7.

2. Zimmermann R, Jakubietz R, Jakubietz M, Strasser E, Schlegel A, Wiltfang J, et al. Different preparation methods to obtain platelet components as a source of growth factors for local application. Transfusion 2001;41(10):1217–24.

3. Whitman DH, Berry RL, Green DM. Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. J Oral Maxillofac Surg 1997;55(11):1294–9.

4. Driver VR, Hanft J, Fylling CP, Beriou JM, Autologel Diabetic Foot Ulcer Study Group. A prospective, randomized, controlled trial of autologous platelet-rich plasma gel for the treatment of diabetic foot ulcers. Ostomy Wound Manage 2006;52(6):68–70.

5. Dugrillon A, Eichler H, Kern S, Kluter H. Autologous concentrated platelet-rich plasma (cPRP) for local application in bone regeneration. Int J Oral Maxillofac Surg 2002;31(6):615–9.

6. Belli E, Longo B, Balestra FM. Autogenous platelet-rich plasma in combination with bovine-derived hydroxyapatite xenograft for treatment of a cystic lesion of the jaw. J Craniofac Surg 2005;16(6):978–80.

7. Smrke D, Gubina B, Domanovic D, Rozman P. Allogeneic platelet gel with autologous cancellous bone graft for the treatment of a large bone defect. Eur Surg Res 2007;39(3):170–4.

8. Currie LJ, Sharpe JR, Martin R. The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review. Plast Reconstr Surg 2001;108(6):1713–26.

9. Horch RE, Bannasch H, Stark GB. Transplantation of cultured autologous keratinocytes in fibrin sealant biomatrix to resurface chronic wounds. Transplant Proc 2001;33(1–2):642–4.

10. Krasna M, Planinsek F, Knezevic M, Arnez ZM, Jeras M. Evaluation of a fibrin-based skin substitute prepared in a defined keratinocyte medium. Int J Pharm 2005;291(1–2):31–7.

11. Mulder GD. Diabetic foot ulcers: old problems – new technologies. Nephrol Dial Transplant 2001;16(4):695–8.

12. Falanga V. The chronic wound: impaired healing and solutions in the context of wound bed preparation. Blood Cells Mol Dis 2004;32(1):88–94.

13. Perseghin P, Sciorelli G, Belotti D, Speranza T, Pogliani EM, Ferro O, Gianoli M, Porta F, Paolini G. Frozen-and-thawed allogeneic platelet gels for treating postoperative chronic wounds. Transfusion 2005;45(9):1544–6.

14. Graziani F, Cei S, Ducci F, Giuca MR, Donos N, Gabriele M. In vitro effects of different concentration of PRP on primary bone and gingival cell lines. Preliminary results. Minerva Stomatol 2005;54(1–2):15–22.

15. Valeri CR, Saleem B, Ragno G. Release of platelet-derived growth factors and proliferation of fibroblasts in the releasates from platelets stored in the liquid state at 22 degrees C after stimulation with agonists. Transfusion 2006;46(2):225–9.

16. Lucarelli E, Beccheroni A, Donati D, Sangiorgi L, Cenacchi A, Del Vento AM, Meotti C, Bertoja AZ, Giardino R, Fornasari PM, Mercuri M, Picci P. Platelet-derived growth factors enhance proliferation of human stromal stem cells. Biomaterials 2003;24(18):3095–100.

17. Kilian O, Flesch I, Wenisch S, Taborski B, Jork A, Schnettler R, Jonuleit T. Effects of platelet growth factors on human mesenchymal stem cells and human endothelial cells in vitro. Eur J Med Res 2004; 9(7):337–44.

18. Crovetti G, Martinelli G, Issi M, Barone M, Guizzardi M, Campanati B, Moroni M, Carabelli A. Platelet gel for healing cutaneous chronic wounds. Transfus Apher Sci 2004;30(2):145–51.

19. Mazzucco L, Medici D, Serra M, Panizza R, Rivara G, Orecchia S, Libener R, Cattana E, Levis A, Betta PG, Borzini P. The use of autologous platelet gel to treat difficult-to-heal wounds: a pilot study. Transfusion 2004;44(7):1013–8.

20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1–2):55–63.

21. Bernas T, Dobrucki J. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 2002;47(4):236–42.

22. Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation. J Oral Maxillofac Surg 2005;63(3):362–9.

23. Chen LB, Buchanan JM. Mitogenic activity of blood components. I. Thrombin and prothrombin. Proc Natl Acad Sci USA 1975;72(1):131–5.

24. Michel D, Harmand MF. Fibrin seal in wound healing: effect of thrombin and [Ca2+] on human skin fibroblast growth and collagen production. J Dermatol Sci 1990;1(5):325–33.

25. Becerra J, Guerado E, Claros S, Alonso M, Bertrand ML, Gonzalez C, Andrades JA. Autologous human-derived bone marrow cells exposed to a novel TGF-beta1 fusion protein for the treatment of critically sized tibial defect. Regen Med 2006;1(2):267–78.

26. Kawaguchi H, Jingushi S, Izumi T, Fukunaga M, Matsushita T, Nakamura T, Mizuno K, Nakamura T, Nakamura K. Local application of recombinant human fibroblast growth factor-2 on bone repair: a dose-escalation prospective trial on patients with osteotomy. J Orthop Res 2007;25(4):480–7.

27. Sugimoto H, Yang C, LeBleu VS, Soubasakos MA, Giraldo M, Zeisberg M, Kalluri R. BMP-7 functions as a novel hormone to facilitate liver regeneration. FASEB J 2007;21(1):256–64.

28. Geer DJ, Swartz DD, Andreadis ST. Biomimetic delivery of keratinocyte growth factor upon cellular demand for accelerated wound healing in vitro and in vivo. Am J Pathol 2005;167(6):1575–86.

29. Persson U, Willis M, Odegaard K, Apelqvist J. The cost-effectiveness of treating diabetic lower extremity ulcers with Becaplermin (Regranex): a core model with an application using Swedish cost data. Value Health 2000;3 Suppl 1:39–46.

30. Akopian G, Nunnery SP, Piangenti J, Rankin P, Rinoie C, Lee E, Alexander M. Outcomes of conventional wound treatment in a comprehensive wound center. Am Surg 2006;72(4):314–7.

31. Nevins M, Giannobile WV, McGuire MK, Kao RT, Mellonig JT, Hinrichs JE, McAllister BS, Murphy KS, McClain PK, Nevins ML, Paquette DW, Han TJ, Reddy MS, Lavin PT, Genco RJ, Lynch SE. Platelet-derived growth factor stimulates bone fill and rate of attachment level gain: results of a large multicenter randomized controlled trial. J Periodontol. 2005;76(12):2205–15.

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