Proliferation-Promoting Effect of Platelet-Rich Plasma on Human Adipose–Derived Stem Cells and Human Dermal Fibroblasts

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Background: This study evaluated changes in platelet-derived growth factor (PDGF)-AB and transforming growth factor (TGF)-β1 release from platelets by platelet-rich plasma activation, and the proliferation potential of activated platelet-rich plasma and platelet-poor plasma on human adipose–derived stem cells and human dermal fibroblasts.
Methods: Platelet-rich plasma was prepared using a double-spin method, with the number of platelets counted in each preparation stage. Platelet-rich and platelet-poor plasma were activated with autologous thrombin and calcium chloride, and levels of platelet-released PDGF-AB and TGF-β1 were determined by enzyme-linked immunosorbent assay. Cells were cultured for 1, 4, or 7 days in serum-free Dulbecco's Modified Eagle Medium supplemented with 5% whole blood plasma, nonactivated platelet-rich plasma, nonactivated platelet-poor plasma, activated platelet-rich plasma, or activated platelet-poor plasma. In parallel, these cells were cultured for 1, 4, or 7 days in serum-free Dulbecco's Modified Eagle Medium supplemented with 1%, 5%, 10%, or 20% activated platelet-rich plasma. The cultured human adipose–derived stem cells and human dermal fibroblasts were assayed for proliferation.
Results: Platelet-rich plasma contained approximately 7.9 times as many platelets as whole blood, and its activation was associated with the release of large amounts of PDGF-AB and TGF-β1. Adding activated platelet-rich or platelet-poor plasma significantly promoted the proliferation of human adipose–derived stem cells and human dermal fibroblasts. Adding 5% activated platelet-rich plasma to the medium maximally promoted cell proliferation, but activated platelet-rich plasma at 20% did not promote it.
Conclusions: Platelet-rich plasma can enhance the proliferation of human adipose–derived stem cells and human dermal fibroblasts. These results support clinical platelet-rich plasma application for cell-based, soft-tissue engineering and wound healing. (*Plast. Reconstr. Surg.* 122: 1352, 2008.)

Autologous platelet-rich plasma has gained in popularity as a clinical treatment in a wide variety of soft- and hard-tissue applications in almost all fields of surgery,[1] particularly in the problematic wound,[2] maxillofacial bone defect,[3] cosmetic surgery,[4,5] and spine literature.[6]

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The usefulness of platelet-rich plasma in the field of plastic and reconstructive surgery has attracted attention.[7] Platelet-rich plasma contains a high concentration of thrombocytes. In α-granules of platelets, there are platelet-released growth factors that include molecules such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, and others, which stimulate cell proliferation and cell differentiation for tissue regen-

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eration. These factors are released from the α-granules in response to platelet activation with platelet aggregation inducers.

Recently, adipose tissue has been confirmed as one source of multipotent adult stem cells,[8,9] and these stem cells are called adipose-derived stem cells. Adipose tissue can be collected under local anesthesia more easily than bone marrow in humans, making the procedure less invasive to the donor; adipose tissue is considered a suitable site for collection of stem cells for clinical application.[10] The multipotent characteristics of human adipose–derived stem cells, and their abundance and accessibility in the human body, make them a potential cell source for tissue engineering applications. Therefore, it is important to increase human adipose–derived stem cell numbers in vitro to obtain a greater number of these cells. In contrast, human dermal fibroblasts play a major role in wound repair. Growth factors released from platelet α-granules have an important impact on the regulation and proliferation of mesenchymal cells, including fibroblasts.[11] We believe that human adipose–derived stem cells and human dermal fibroblasts should be counted among the cells that will play an important role in tissue engineering in the future of plastic surgery.

To date, studies investigating the effect of platelet-rich plasma on cell function in vitro, which will provide important data for clinical application, have used a wide variety of cell types and obtained conflicting results.[12–18] However, little has been reported regarding the effect of the platelet-rich plasma concentration on the proliferation of human adipose–derived stem cells and human dermal fibroblasts. To clarify the clinical usefulness of platelet-rich plasma, with the evaluation of PDGF, the effect of platelet-rich plasma on the proliferation of human adipose–derived stem cells and human dermal fibroblasts needs to be investigated. The objective of this study was to evaluate changes in PDGF-AB and TGF-β1 release from platelets by platelet-rich plasma activation and, moreover, investigate the effect of different platelet-rich plasma concentrations on the proliferation of human adipose–derived stem cells and human dermal fibroblasts.

MATERIALS AND METHODS

Cell Culture

Human abdominal subcutaneous fat was collected from excess tissues excised during plastic and reconstructive surgery. Human adipose–derived stem cells were prepared as described previously.[8] Briefly, adipose tissue was washed extensively with 20 ml of phosphate-buffered saline three times and cut into small pieces. Then, the extracellular matrix was digested with 0.1% collagenase solution with shaking at 37°C for 40 minutes. After adding basal medium consisting of Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, and 1% penicillin, it was centrifuged at 1500 rpm for 3 minutes. After removing cellular remains through a 100-μm nylon mesh, the cells were incubated in control medium in a dish. The adhered human adipose–derived stem cells were maintained until passage 3 in control medium.

Human dermal fibroblasts were harvested from the same above-mentioned patient as described previously.[19,20] Cells were maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% penicillin. All subjects enrolled in this research gave informed consent. This study was approved by our institutional committee on human research, which found this protocol to be acceptable.

Preparation of Activated Platelet-Rich Plasma

Platelet-rich plasma was prepared using a platelet-rich plasma kit (JP200; Japan Paramedeic Co., Ltd, Tokyo, Japan). Briefly, blood was collected into tubes containing acid-citrate-dextrose solution formula A (1:4 vol/vol) anticoagulant, from healthy adult volunteers ($n = 5$) after receiving informed consent. The citrated blood was centrifuged in a standard laboratory centrifuge for 7 minutes at 1700 rpm. Subsequently, the yellow plasma with buffy coat (containing the platelets, leukocytes, and a few erythrocytes) was taken up into a Monovette (Sarstedt, Inc., Newton, N.C.) with a long cannula. The second centrifugation was performed for 5 minutes at 3200 rpm. The platelet pellet accumulated at the bottom and the platelet-poor plasma accumulated on top. The plasma supernatant was used as platelet-poor plasma, and the thrombocyte pellet in 1.0 ml of plasma was used as platelet-rich plasma. Before and after preparation of platelet-rich plasma, an aliquot was removed and platelets were counted.

A 1:1 (vol/vol) mixture of 0.5 M calcium chloride and autologous thrombin was prepared in advance as an activator. A 10:1 (vol/vol) mixture of platelet-rich plasma or platelet-poor plasma and the activator was incubated for 5 minutes at room temperature, and this mixture was regarded as activated platelet-rich plasma or activated platelet-poor plasma (Figs. 1 and 2). Nonactivated whole blood plasma, nonactivated platelet-rich plasma,

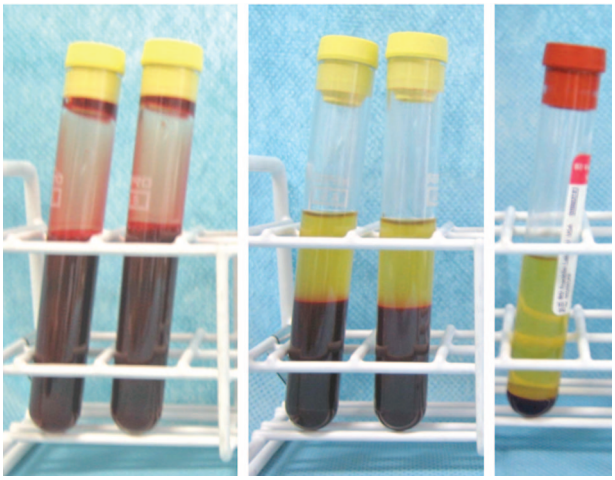

Fig. 1. Method of platelet-rich plasma preparation, part I. Platelet-rich plasma was prepared according to the manufacturer's instructions. Whole blood (*left*) was centrifuged at 1700 rpm for 7 minutes (*center,* first spin), and the supernatant was transferred to another conical tube, which was centrifuged at 3200 rpm for 5 minutes (*right,* second spin).

Fig. 2. Method of platelet-rich plasma preparation, part II. The resulting supernatant was platelet-poor plasma (*above, left*), and 1 ml of the bottom layer (i.e., 1 ml of platelet-poor plasma containing the buffy coat) was regarded as platelet-rich plasma (*below, left*). The addition of the activator to platelet-rich plasma (*below, right*) or platelet-poor plasma (*above, right*) caused it to clot, and caused the release of PDGF and TGF-β from the platelets.

nonactivated platelet-poor plasma, activated platelet-rich plasma, and activated platelet-poor plasma were centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored at −20°C until used.

Determination of TGF-β and PDGF Levels in Platelet-Rich Plasma

The TGF-β1 and PDGF-AB levels in the preparation of nonactivated platelet-rich plasma, activated platelet-rich plasma, nonactivated platelet-poor plasma, activated platelet-poor plasma, and whole blood were determined by a commercially available sandwich enzyme-linked immunosorbent assay technique kit (Quantikine; R&D Systems, Inc., Minneapolis, Minn.). Growth factor concentrations were measured according to the manufacturer's instructions.

Cell Proliferation Assay

Adipose-derived stem cells and dermal fibroblasts were seeded at a density of 3.0×10^2 cells/well in 96-well culture plates. The cells were cultured after the addition of serum-free Dulbecco's Modified Eagle Medium supplemented with 5% whole blood, nonactivated platelet-rich plasma, nonactivated platelet-poor plasma, activated platelet-rich plasma, or activated platelet-poor plasma for 1, 4, or 7 days. In parallel, the cells were cultured after the addition of serum-free Dulbecco's Modified Eagle Medium supplemented with 0% (control), 1%, 5%, 10%, or 20% activated platelet-rich plasma for 1, 4, or 7 days. The cultured cells were assayed for proliferation using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, Md.). Cell numbers were measured according to the manufacturer's instructions. The rationale for the cell counting assay using the Cell Counting Kit-8 and the precautions to be taken on adding platelet-rich plasma were elaborated on. The color-developing substrate WST-8 contained in the Cell Counting Kit-8 is reduced by intracellular dehydrogenase to water-soluble formazan. The amount of formazan in a dish can be directly measured photometrically at 450 nm. The cell count has been shown to be linearly proportional to the amount of formazan generated. Thus, in a preliminary experiment, standard curves for human adipose–derived stem cell and human dermal fibroblast counts versus absorbance were constructed, and then the cell count was determined from the absorbance of the test sample. Whole blood plasma, nonactivated platelet-rich plasma, nonactivated platelet-poor plasma, activated platelet-rich plasma, and activated platelet-poor plasma were centrifuged at 10,000 rpm for 15 minutes, and only the supernatant was added to the culture medium, with sufficient care to prevent the addition of blood cell components.

Statistical Analysis

The Mann-Whitney *U* test was used for comparison between groups, with values of $p < 0.05$ being regarded as significant. Data are presented as means ± SD.

RESULTS

Platelet Counts and Levels of Growth Factors

Platelet counts in whole blood, platelet-rich plasma, and platelet-poor plasma showed means of 16.74×10^4 cells/μl, 132.26×10^4 cells/μl, and 5.12×10^4 cells/μl, respectively (Fig. 3). The concentration of platelets in platelet-rich plasma was approximately 7.9-fold the concentration in whole blood.

Supernatant of activated platelet-rich plasma contained high levels of PDGF-AB and TGF-β1 (Fig. 4). The mean PDGF-AB level in nonactivated platelet-rich plasma was 0.773 ± 0.83 pg/ml and became concentrated 184-fold to 144.46 ± 24.27 pg/ml. In contrast, The mean TGF-β1 level in nonactivated platelet-rich plasma was 0.982 ± 0.15 pg/ml and became concentrated 81-fold to 96.38 ± 16.77 pg/ml.

Effect of Platelet-Rich Plasma and Platelet-Poor Plasma on Proliferation of Human Adipose–Derived Stem Cells and Human Dermal Fibroblasts

The effects of whole blood plasma, nonactivated platelet-rich plasma, activated platelet-rich plasma, nonactivated platelet-poor plasma, and activated platelet-poor plasma on human adipose–derived stem cells and human dermal fibroblasts are shown in Figure 5. Close to a 2.5- to 3-fold increase in the number of human adipose–derived stem cells and human dermal fibroblasts was observed in activated platelet-rich plasma and activated platelet-poor plasma groups compared with the before-activation groups (nonactivated platelet-rich plasma and nonactivated platelet-poor plasma). Among the five groups, activated platelet-rich plasma had the strongest proliferative effect on human adipose–derived stem cells and human dermal fibroblasts (Fig. 5).

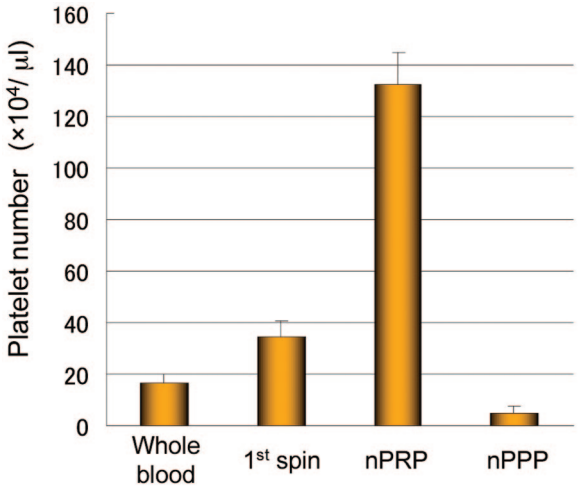

Fig. 3. Descriptive statistics for the platelet counts in the respective blood fractions of the different preparations ($n = 5$). The concentration of platelets in platelet-rich plasma increased by approximately 7.9-fold. *nPRP,* nonactivated platelet-rich plasma; *nPPP,* nonactivated platelet-poor plasma.

Effect of Activated Platelet-Rich Plasma Concentrations on Proliferation of Human Adipose–Derived Stem Cells and Human Dermal Fibroblasts

The proliferation of human adipose–derived stem cells peaked on day 7 of culture in the presence of 1% or 5% activated platelet-rich plasma and decreased in a dose-dependent manner in the presence of 10% or 20% activated platelet-rich plasma (Fig. 6). The proliferation of human dermal fibroblasts peaked on day 7 of culture in the presence of 5% activated platelet-rich plasma and decreased in a dose-dependent manner in the presence of 10% or 20% activated platelet-rich plasma. Cell proliferation was little observed in the serum-free controls, but cell proliferation markedly increased in the presence of activated platelet-rich plasma on day 4 of culture. The number of human dermal fibroblasts decreased on day 1 of culture in the presence of 10% or 20% activated platelet-rich plasma ($n = 5$). Data represent the means of all experimental runs.

DISCUSSION

This study showed that activated platelet-rich plasma contained large amounts of PDGF-AB and TGF-β1 and that their addition to the culture medium promoted the proliferation of human adipose–derived stem cells and human dermal fibroblasts. The addition of 5% activated platelet-rich plasma to the culture medium maximally promoted cell proliferation, but higher concentrations of activated platelet-rich plasma did not promote it. This suggests that activated platelet-rich plasma is useful in tissue engineering using human adipose–derived stem cells and human dermal fibroblasts, and that it is necessary to control the release of growth factors from platelets at appropriate concentrations.

Platelet-rich plasma is a concentration of platelets in a small volume of plasma. Platelet α-gran-

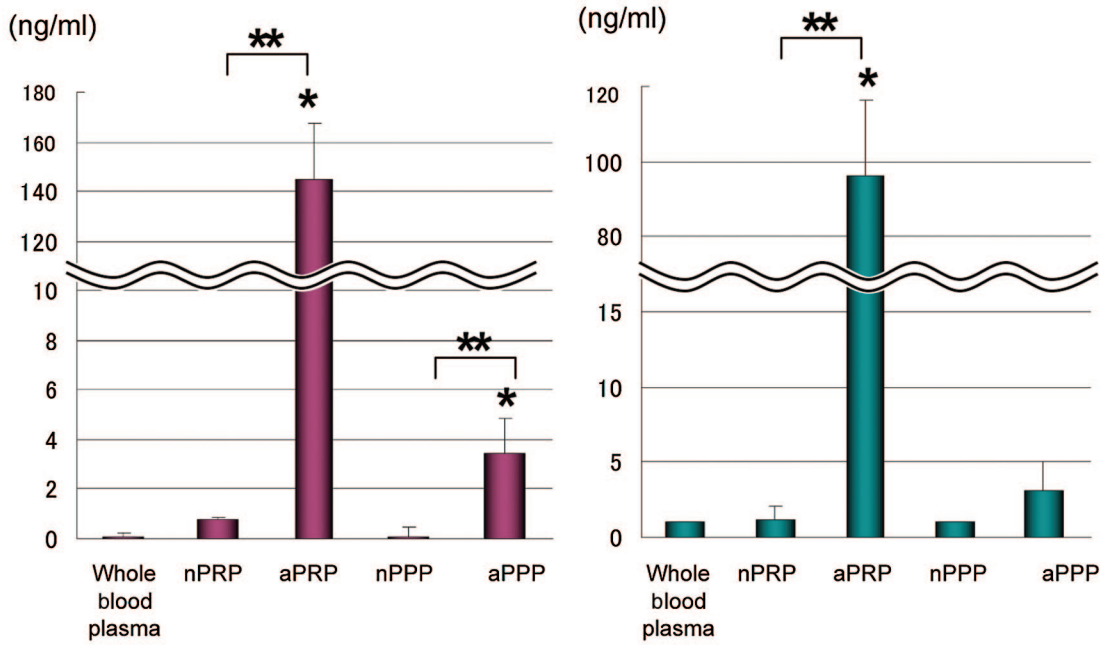

Fig. 4. Changes in PDGF-AB (*left*) and TGF-β1 (*right*) levels before and after platelet activation. The levels of PDGF-AB and TGF-β1 after platelet-rich plasma activation were 187 times (mean, 144.46 ± 24.27 ng/ml) and 81 times (mean, 96.38 ± 16.77 ng/ml), respectively, higher than those before platelet-rich plasma activation ($n = 5$). *$p < 0.05$ versus whole blood plasma; **$p < 0.05$ before activation versus after activation. *nPRP,* nonactivated platelet-rich plasma; *aPRP,* activated platelet-rich plasma; *nPPP,* nonactivated platelet-poor plasma; *aPPP,* activated platelet-poor plasma.

ules contain growth factors such as PDGF, TGF-β, insulin-like growth factor, epidermal growth factor, and others. PDGF is a powerful mitogen for fibroblasts and smooth muscle cells and is involved in all three phases of wound healing, including angiogenesis, the formation of fibrous tissue, and reepithelialization.[21] PDGF is composed of two polypeptide chains (A and B) combined in three disulfide-linked dimeric forms (AA, AB, and BB).[22] Human platelets have all three PDGF dimers, in quantities of approximately 65 percent AB, 23 percent BB, and 12 percent AA. In this study, PDGF-AB was evaluated because of the majority in the three dimers. In contrast, the TGF-β family of proteins is the most recent family to be discovered.[23] A distinguishing characteristic of the TGF-β family of proteins is their ability to reversibly inhibit the growth of a number of cell types, particularly cells derived from the ectoderm such as keratinocytes and leukocytes.[24] However, it has been reported that TGF-βs are weak mitogens for cells derived from the mesoderm, such as fibroblasts. Three distinct TGF-βs have been identified in humans: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 was initially isolated and sequenced from platelets and, like TGF-α, was named for its ability to reversibly stimulate the growth of normal fibroblasts in soft agar.[25] Therefore, we measured the levels of PDGF-AB and TGF-β1 in platelet-rich plasma among the PDGFs and TGF-βs.

Enzyme-linked immunosorbent assay was used to quantitate profile growth-factor release from the platelet-rich plasma collected. Weibrich et al. recently evaluated concentrated levels of PDGF-AB and TGF-β1 in platelet-rich plasma obtained from the same whole blood sample by two different methods.[26] PDGF-AB levels were determined to be 133 ng/ml and 233 ng/ml, respectively, and TGF-β1 levels were 268 ng/ml and 95 ng/ml, respectively. Okuda et al. reported that PDGF-AB and TGF-β1 levels in the platelet-rich plasma were 182.0 ng/ml and 140.9 ng/ml, respectively.[27] Thus, the levels of growth factors in platelet-rich plasma varied according to the method of platelet-rich plasma preparation. In this study, a 1-ml platelet-rich plasma sample contained approximately 13.23×10^5 platelets, and PDGF-AB and TGF-β1 levels were 144.46 ± 24.27 ng/ml and 96.38 ± 16.77 ng/ml, respectively. We prepared platelet-rich plasma by a double-spin method for the manual separation of blood cell layers, and found that centrifugation resulted in a 7.9-fold higher con-

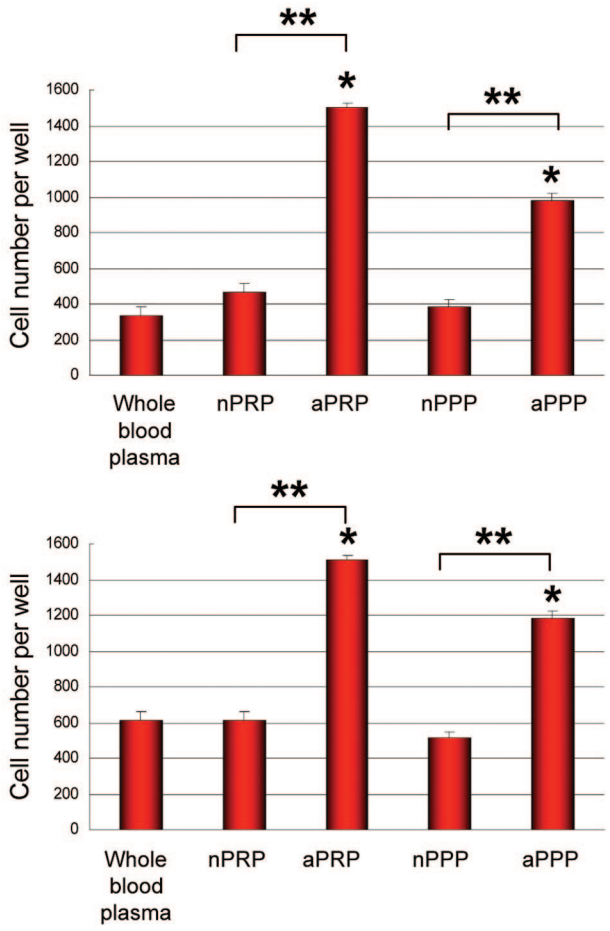

Fig. 5. Human adipose–derived stem cell (*above*) and human dermal fibroblast (*below*) proliferation on day 7 after whole blood plasma, nonactivated platelet-rich plasma (*nPRP*), nonactivated platelet-poor plasma (*nPPP*), activated platelet-poor plasma (*aPRP*), and activated platelet-poor plasma (*aPPP*) (5%) additions. The addition of activated platelet-rich plasma maximally promoted the proliferation of human adipose–derived stem cells and human dermal fibroblasts. *$p < 0.05$ versus whole blood plasma; **$p < 0.05$ before activation versus after activation ($n = 5$).

centration of platelets in platelet-rich plasma than that in whole blood plasma, and PDGF-AB and TGF-β1 were concentrated 187- and 81-fold, respectively. Some studies reported a positive correlation between platelet counts and growth factor levels,[28–31] whereas other studies found no correlation between them[7,32]; no conclusions have been reached. In this study, the activation of platelet-rich plasma and platelet-poor plasma was associated with the release of large amounts of PDGF-AB and TGF-β1, and their addition to human adipose–derived stem cells and human dermal fibroblasts markedly promoted their proliferation. In contrast, the addition of nonactivated platelet-rich plasma or platelet-poor plasma did not promote cell proliferation, compared with controls. These results mean that the activation of platelet-rich plasma and platelet-poor plasma is essential for their use.

Activated platelet-poor plasma is mitogenic for a variety of cell types, such as human mesenchymal progenitor cells,[13,14] AG1518 fibroblasts,[10] bone cells from adults,[33] rat bone marrow-derived stem cells,[34] mesenchymal stem cells from adipose tissue,[35] endothelial cells,[36] rat calvarial bone cells,[37] calf periosteum-derived cells,[38] dog alveolar bone cells,[39] and human osteoblasts and gingival fibroblasts.[40] In contrast, activated platelet-poor plasma is mitogenic for primary human adipocytes.[41] However, few data exist with regard to human adipose–derived stem cells and human dermal fibroblasts. Graziani et al. reported that the maximum effect of activated platelet-rich plasma was achieved in a concentration of 2.5× platelet-rich plasma, with higher concentrations resulting in a reduction of cell proliferation.[40] Choi et al. examined the influence of platelet-rich plasma concentrations on the viability and proliferation of alveolar bone cells, and the results showed that the viability and proliferation of alveolar bone cells were suppressed by high platelet-rich plasma concentrations but were stimulated by low platelet-rich plasma concentrations (1% to 5%).[39] Our studies have indicated that activated platelet-rich plasma also stimulated the proliferation of human adipose–derived stem cells and human dermal fibroblasts, which is consistent with the findings reported above. In addition, the results showed that 1% and 5% were suitable concentrations for human adipose–derived stem cells proliferation, and 5% was a suitable concentration for human dermal fibroblasts. Therefore, dilution of the platelet-rich plasma concentration to a level suitable for its cell proliferation may be necessary for use in regenerative medicine to enhance human adipose–derived stem cell and human dermal fibroblast growth through platelet-rich plasma application. Reasons for the differences in concentrations of platelet-rich plasma suitable for human adipose–derived stem cells and human dermal fibroblasts are as follows: 1% and 5% platelet-rich plasma were suitable for the growth of human adipose–derived stem cells, and 5% platelet-rich plasma was suitable for that of human dermal fibroblasts. Human adipose–derived stem cells are pluripotential stem cells of the body, whereas human dermal fibroblasts are mature fibroblasts derived from the dermis. The difference in the degree of cell differentiation may have influenced the sensitivity of platelet-rich plasma to cytokines,

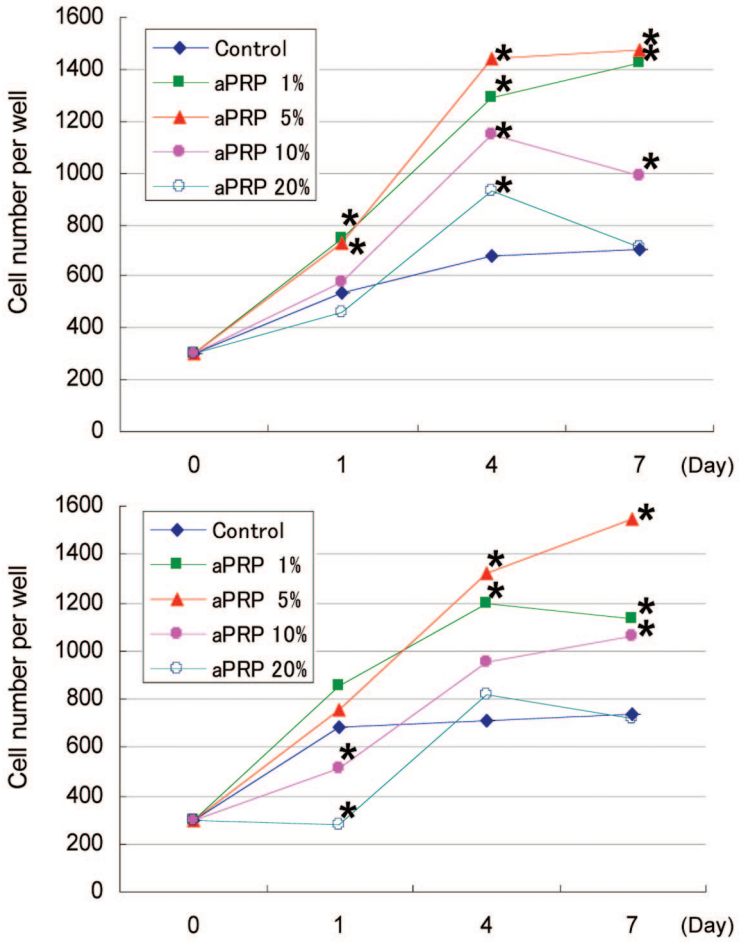

Fig. 6. Human adipose–derived stem cell (*above*) and human dermal fibroblast (*below*) proliferation on days 1, 4, and 7 after the addition of various concentrations of activated platelet-rich plasma (*aPRP*). The addition of 5% activated platelet-rich plasma to the culture medium maximally promoted cell proliferation, but activated platelet-rich plasma at 20% did not promote it. *$p < 0.05$ versus control ($n = 5$).

leading to differences in the appropriateness of concentrations. From now on, not only for hard-tissue engineering but also for soft-tissue engineering and promotion of wound-healing, platelet-rich plasma could be more widely and usefully applied. Human dermal fibroblasts are the main components of the skin and subcutaneous tissue, and human adipose–derived stem cells are a source of stem cells in the body, differentiating into adipocytes, chondrocytes, and osteocytes. This study revealed the proliferative effect of platelet-rich plasma on human dermal fibroblasts and human adipose–derived stem cells, suggesting its potential for the promotion of wound healing and remodeling of the skin and soft tissue. The clinical application of autologous blood–derived platelet-rich plasma may be very useful in promoting wound healing and skin and soft-tissue remodeling without the risk of immune rejection or infection. Further investigations into the platelet-rich plasma concentration/effect relationship in vivo should be conducted to clarify whether or not the results of this study are in accordance with the clinical findings.

CONCLUSIONS

We confirmed that platelet-rich plasma contained approximately 7.9 times as many platelets as whole blood, and that platelet-rich plasma activation was associated with the release of large amounts of PDGF-AB and TGF-β1. The addition of activated platelet-rich plasma or activated platelet-poor plasma significantly promoted the proliferation of adipose-derived stem cells and dermal

fibroblasts. The addition of 5% activated platelet-rich plasma to the medium maximally promoted cell proliferation, but activated platelet-rich plasma at 20% did not promote it. From now on, not only for hard-tissue engineering but also for soft-tissue engineering and promotion of wound healing, platelet-rich plasma could be more widely and usefully applied.

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