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Platelet-rich plasma inhibits the apoptosis of highly adipogenic homogeneous preadipocytes in an *in vitro* culture system

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Abbreviation: BIM, Bcl-2-interacting mediator of cell death

Abstract

Auto-transplantation of adipose tissue is commonly used for the treatment of tissue defects in plastic surgery. The survival of the transplanted adipose tissue is not always constant, and one of reasons is the accelerated apoptosis of the implanted preadipocytes. We have recently established highly homogeneous preadipocytes, named ccdPAs. The aim of the current study was to evaluate the regulation of the potency of platelet-rich plasma (PRP) on the apoptosis of ccdPAs *in vitro*. PRP stimulated the proliferation of the preadipocytes in a dose-dependent manner, and the stimulatory activity of 2% PRP was significantly higher than that of 2% FBS or 2% platelet-poor plasma (PPP). The presence of 2% PRP significantly inhibited serum starvation- or TNF-α/cycloheximide-induced apoptosis in comparison to 2% FBS or 2% PPP. DAPK1 and Bcl-2-interacting mediator of cell death (BIM) mRNAs were reduced in the preadipocytes cultured with 2% PRP in comparison to those cultured in 2% FBS. The gene expression levels were significantly higher in cells cultured without serum in comparison to cells cultured with 2% FBS, and the levels in the cells with 2% PRP were reduced to 5-10% of those in the cells without serum. These results indicated that ccdPAs exhibit anti-apoptotic activities, in addition to increased proliferation, when cultured in 2% PRP in comparison to the same concentration of FBS, and that this was accompanied with reduced levels of DAPK1 and BIM mRNA expression in *in vitro* culture. PRP may improve the outcome of transplantation of adipose tissue by enhancing the anti-apoptotic activities of the implanted preadipocytes.

Keywords: adipocytes; apoptosis; Bcl-2-like protein 11; death-associated protein kinase; platelet-rich plasma; tissue transplantation

Introduction

Aspirated fat is a common source of autologous tissue transplantation for the correction of tissue defects in plastic and reconstructive surgery (Billings and May, 1989; Patrick, 2000, 2001). Aspirated fat contains multipotential preadipocytes and progenitor cells, which have been utilized as a source of cell-based regenerative medicine (Stashower *et al*., 1999; Zuk *et al*., 2001; Gimble *et al*., 2007; Yoshimura *et al*., 2009; Bauer-Kreisel *et al*., 2010; Sterodimas *et al*., 2010). Although several different techniques of fat grafting have been developed, the outcomes of the transplantation vary widely. The most important factor required for successful grafting is to optimize the survival of the transplanted preadipocytes and other cells in the graft. In previous studies, we and others have shown that various cytokines are involved in the efficient cell survival of the implants (Kimura *et al*., 2003;

Yamaguchi *et al*., 2005; Cho *et al*., 2006; Torio-Padron *et al*., 2007; Kuramochi *et al*., 2008; Ning *et al*., 2009).

Platelet rich plasma (PRP) (Eppley *et al*., 2006; Foster *et al*., 2009; Redler *et al*., 2011) has been widely applied for practical medicine, such as aesthetic plastic surgery and the treatment of soft-tissue ulcers (Welsh, 2000; Man *et al*., 2001; Margolis *et al*., 2001; Bhanot and Alex, 2002; Martinez-Zapata *et al*., 2009; Sclafani, 2009). Once activated, platelets secrete various bioactive cytokines, including platelet-derived growth factor (PDGF) and transforming growth factor beta 1 (TGF-β1), which increase angiogenesis and cell proliferation relevant to soft tissue regeneration. PRP has been applied for fat grafting, and in fact, has been shown to improve the survival of implanted adipose tissue in patients (Abuzeni and Alexander, 2001; Sadati *et al*., 2006; Cervelli *et al*., 2009). Thus, the use of PRP has been broadened to the tissue-engineering field using adipose tissue-derived multi-potential cells (Anitua *et al*., 2006; Muller *et al*., 2009). PRP is also expected to function as an autologous fibrin-based scaffold for transplanted cells (Anitua *et al*., 2006; Wu *et al*., 2009; Kang *et al*., 2011). In fact, recent our study showed that fibrin-based scaffold decreased the apoptotic cell death of murine ccdPAs in mice transplantation model (Aoyagi *et al*., 2011).

We have recently identified proliferative preadipocytes, ceiling culture-derived proliferative adipocytes (ccdPAs), as homogeneous cells suitable for *ex vivo* gene therapy applications *via* autologous transplantation (Asada *et al*., 2011; Kuroda *et al*., 2011). The ccdPAs are characterized by their high proliferative capacity with spontaneous adipogenic potential in scaffold fibrin gel culture (Aoyagi *et al*., 2012). The establishment of a highly homogenous preadipocyte line made it possible to perform examinations to identify the optimal scaffolds and cytokines that can be used to improve the survival of transplanted preadipocytes. We herein studied the effects of PRP, and an autologous cytokine cocktail, on the apoptotic properties of preadipocytes using the ccdPAs.

Results

PRP inhibits fibrin scaffold gel shrinkage and improves the viability of ccdPAs in 3-dimensional culture

We have recently established a 3-dimensional (3-D) culture system for ccdPAs using fibrin gel (FG) (Aoyagi *et al*., 2012). Using the 3-D culture system, the effects of PRP on the gel shrinkage and cell viability were analyzed in comparison to FBS. The FG/ccdPAs were formed and maintained in culture medium containing 10% FBS for 16 hr. The culture medium was replaced with fresh medium containing 2% PRP, 2% FBS or 10% FBS, or with medium without serum, and cells were subsequently incubated for an additional 24 hr.

The resulting gel sizes varied among the cultures grown in each type of medium. The gels without serum or with 2% FBS showed a drastic volume reduction, while the volumes of the gels cultured with 10% FBS or 2% PRP were not obviously reduced (Figure 1A). The culture supernatants were collected from each well and LDH activity was measured to evaluate the viability of cells. The LDH activity significantly decreased in the culture medium with 2% PRP in comparison to the medium with 2% or 10% FBS (Figure 1B). TUNEL staining of the gel sections showed the number of apoptotic cells to significantly decrease in the medium with 2% PRP in comparison to the medium with 2% FBS (1.5 ± 1.1% vs 9.8 ± 1.9%, $P < 0.05$). These results suggested that 2% PRP inhibits the shrinkage of FG/ccdPAs gels, and improves the cell viability in comparison to the same concentration of FBS.

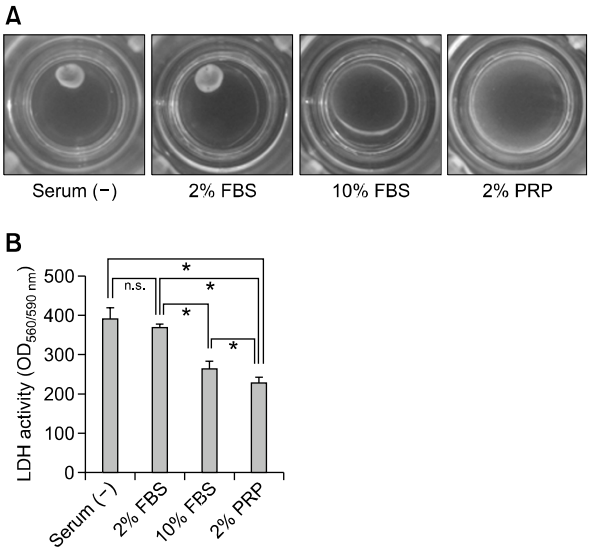

Figure 1. The effects of platelet-rich plasma (PRP) on the 3-dimensional culture of ccdPAs. 100 μl of fibrin gels containing 1×10^7 cells/ml of ccdPAs (FG/ccdPAs) were formed in cell culture insert and incubated in DMEM/HAM with 10% FBS for 16 hrs. The medium was replaced by DMEM/HAM in the presence or absence of different concentrations of FBS or PRP. (A) Photographs of FG/ccdPAs in the inserts were taken after 24 hr of culture. (B) The culture supernatant was collected from each well and the LDH activities expressed by the fluorescence of resorufin generated by coupled enzymatic reaction were examined. *$P < 0.05$.

PRP has a high proliferation-inducing potential for ccdPAs in plate culture

In order to evaluate the function of PRP on cell survival in the gel, we next examined the effects of PRP on the proliferation of ccdPAs in comparison to FBS. The cells (2.5×10^5 cells) were seeded and incubated with DMEM/HAM containing 20% FBS in 10 cm dishes for 16 hr. The media was replaced with medium containing 2% PRP, 2% FBS, or 10% FBS, and the cells were then cultured for 3 days. The cell appearance was not apparently changed among the ccdPAs cultured for 3 days in plates with media containing 2% PRP, 2% FBS, or 10% FBS (Figure 2A). To examine the cell proliferation, 2×10^3 cells of ccdPAs were seeded onto 96 well plates and incubated at 37°C for 24 hr. The media was replaced with medium with or without 2% PRP, 2% FBS, or 10% FBS (Day 0), and the cells were cultured for 3 days. The number of cells in each well was evaluated by measuring the DNA content. In contrast to the observation that the cell numbers on Day 3 were not significantly changed in comparison to those at Day 0 in the cultures incubated in medium containing 2% FBS, the cell numbers were significantly increased in cells cultured in the medium with 10% FBS or 2% PRP, and notably, the number of cells on Day 3 in the medium containing 2% PRP was significantly increased in comparison to the cells cultured with 10% FBS (Figure 1B). The cell numbers in the media with various concentrations of PRP showed a dose-dependent increase up to 5% PRP; the number of cells present in the media with 0.5-1% PRP was almost equivalent to that of the cells cultured with 5-10% FBS (Figure 2C). These results indicated that the proliferation-inducing potential of PRP for ccdPAs was higher in comparison to that of FBS.

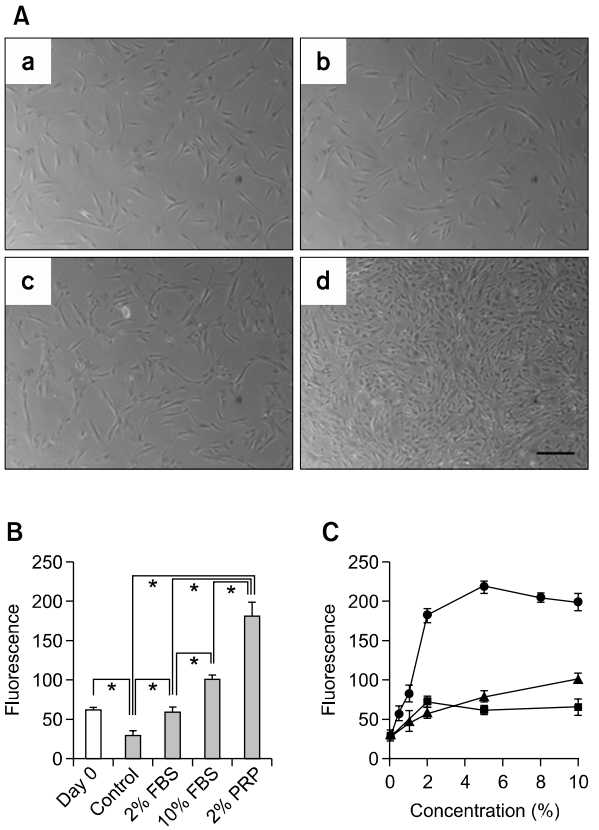

Figure 2. Platelet-rich plasma (PRP) promotes the proliferation of ccdPAs. (A) Photographs of ccdPAs were taken after 3 days in culture containing DMEM/F12-HAM in the absence of serum (a) or the presence of 2% (b) or 10% (c) FBS or 2% (d) PRP. The scale bar indicates 200 μm. (B) Cells were seeded into 96 well plates and incubated in DMEM/F12-HAM containing 20% FBS for 24 hr. The culture medium was then replaced with DMEM/F12-HAM in the absence of serum (control) or presence of 2% FBS or 10% FBS or 2% PRP. After 3 days in CO_2 incubator, the cell number in each well was examined by DNA content in comparison to those on day 0 (open bar). The values for the serum (-), 10% FBS, and 2% PRP groups were significantly different compared with those on Day 0 (*$P < 0.05$). (C) The cell numbers were examined in wells cultured in DMEM/F12-HAM containing various concentrations of PRP (circle), FBS (triangle), and PPP (square) after 3 days of culture similarly as Figure 2B. The growth stimulatory effect was not significantly different between 10% and 20% FBS and PPP (data not shown).

PRP inhibits the apoptosis of ccdPAs

The high proliferation-inducing property of PRP for ccdPAs in culture prompted us to further analyze PRP for protective effects against apoptosis in ccdPAs, since PRP is rich in cytokines and proteinases involved not only in proliferation, but also in regulating apoptosis (Eppley *et al*., 2004, 2006; Foster *et al*., 2009; Redler *et al*., 2011). The protection of the cells from death may contribute to their longer survival after transplantation, together with a high potential for proliferation. To investigate the possibility, the ccdPAs (1×10^6 cells) were seeded and incubated in a 10 cm dish with DMEM/HAM medium containing 20% FBS for 16 hr, and subsequently incubated with the medium with or without 2% FBS, 2% PRP, or 2% PPP. After incubation for 8 hr, the cells were collected and stained with Annexin V-FITC. The flow cytometric analysis showed that 5.5% of cells were identified as Annexin V positive in the medium without serum (Figure 3A). The number of apoptotic cells was significantly decreased in the media with 2% FBS, 2% PRP, or 2% PPP in comparison to that in the cells cultured in serum-free medium. Among the various supplements, 2% PRP drastically reduced the number of apoptotic cells in comparison to 2% FBS or 2% PPP. ERK1/2 phosphorylation was examined to further analyze the protective effect of PRP against apoptosis, because the activation of the

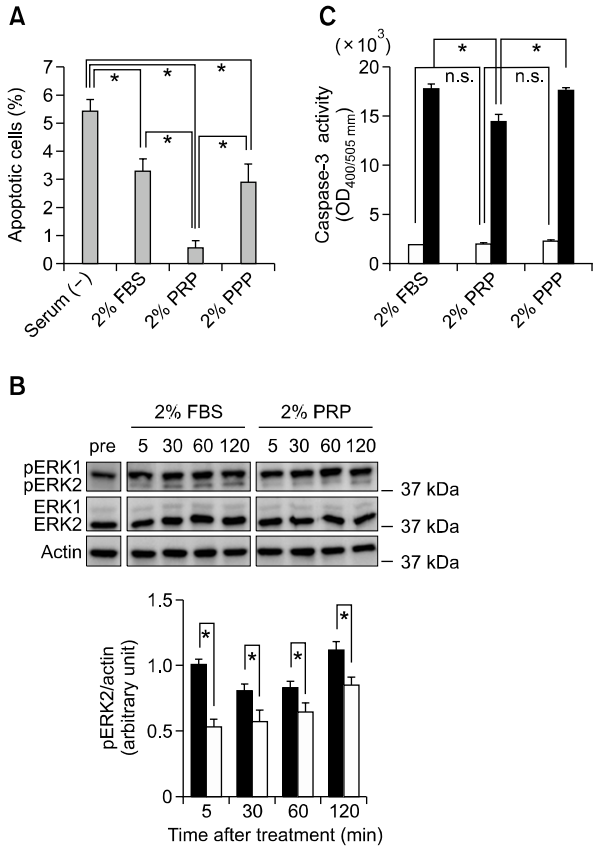

Figure 3. PRP decreased the apoptotic cell death and caspase 3 activity induced by TNF-α and cycloheximide in ccdPAs. (A) ccdPAs were seeded and incubated for 16 hr with medium containing 20% FBS, and the medium was then replaced with fresh medium without serum (Serum (-)) or with media containing 2% PRP, FBS, or PPP, and the cells were subsequently incubated at 37°C for 8 hr. The cells were then collected, stained by Annexin V-FITC and propidium iodide (PI), and analyzed using a Tali™ Image Based Cytometer (Life technologies). FITC-positive/PI-negative cells were considered to be apoptotic cells. *$P < 0.05$. (B) After incubation for 16 hr with medium containing 20% FBS, ccdPAs were pretreated for 2 hr with the medium containing 2% PRP or FBS. The cells were treated with TNF-α and cycloheximide for 5, 30, 60, and 120 min in the medium with 2% FBS (closed bar) or 2% PRP (open bar). Cell lysates were prepared at each time point as well as pretreatment (pre) and then were subjected to an immunoblot analysis of phosphorylated or unphosphorylated form of ERK1/2, and Actin. Results of densitometric analysis of phosphorylated form of ERK2 (pERK2) is shown below. After normalization of signals of pERK2 by Actin, each value of pERK2 was expressed as the fold increase of that at 5 min with 2% FBS. *$P < 0.05$. (C) After pretreatment for 2 hr with the medium containing 2% PRP, FBS, or PPP, the cells were incubated for 3 hr in the absence (open bars) or presence (closed bars) of TNF-α and cycloheximide, which induce apoptotic cell death. The caspase-3 activity in the lysates of collected cells were measured. *$P < 0.05$.

cascade is important for apoptosis *via* various intracellular signals including TNF-α (Cawthorn and Sethi, 2008; Mebratu and Tesfaigzi, 2009; Cagnol and Chambard, 2010). The cells were incubated with 2% PRP, FBS, or PPP for 2 hr, and apoptosis was induced by TNF-α and cycloheximide. Phosphorylation was detected following treatment with TNF-α and cycloheximide for 5 min in the cells cultured with 2% FBS. A densitometric analysis showed the amount of phosphorylated ERK2, and not phosphorylated ERK1, to significantly decrease in the medium with 2% PRP in comparison to the medium with 2% FBS (Figure 3B). The caspase-3 activity induced by TNF-α and cycloheximide for 3 hr were also significantly decreased in ccdPAs in the medium with 2% PRP in comparison to the cells cultured with 2% FBS or 2% PPP (Figure 3C). Thus, the apoptosis of ccdPAs was inhibited by culturing them in the medium with 2% PRP *in vitro*.

PRP almost completely inhibits the expression of the pro-apoptotic genes, DAPK1 and BIM, in ccdPAs after serum starvation

In order to identify the molecules involved in the anti-apoptotic effects of PRP on ccdPAs in culture, the expression profiles of representative apoptosis-related genes were examined using a PCR array profiler. The cells (2.5×10^5 cells) were seeded into 10 cm dishes and incubated in DMEM/HAM containing 20% FBS for 16 hr. The media was replaced with DMEM/HAM containing 2% PRP or FBS, the cells were incubated for 3 days, and the total RNA was isolated from the cultured cells to analyze the expression of apoptosis-related genes. Two independent experiments showed that, among the 84 genes examined, there were 8 genes with a more than 2-fold increase in expression, and 9 genes with a more than 2-fold decrease in expression in the cells cultured in the medium with 2% PRP compared with those cultured in the medium with 2% FBS (Table 1). We focused our interest on two genes, DAPK1 (reduced to 7.4% of the expression level observed with FBS) and BCL2L11 (also called BIM, reduced to 18.9% of the level observed with FBS), as representative genes with the obvious downregulation in the medium with 2% PRP (Figure 4). DAPK1 and BIM have been shown to be one of master regulators of cell death (Gozuacik and Kimchi, 2006), and is essential for BAX-dependent cell death (Kim *et al*., 2009; Ren *et al*., 2010), respectively.

The role of DAPK1 and BIM genes in apoptosis of preadipocytes was investigated by examining the effect of serum starvation of cells incubated with PRP on the expressions of these genes. The ccdPAs (2.5×10^5 cells) were seeded into 10 cm dishes and incubated in DMEM/HAM containing 20% FBS for 16 hr. The medium was replaced by medium containing 10% FBS (control), 2% FBS, 2% PRP, or 2% PPP, the cells were cultured for 3 days and the expression of DAPK1 and BIM were

Table 1. Apoptosis-related genes affected by PRP

Unigene	Refseq	Symbol	Description	Fold change
Downregulated				
Hs.380277	NM_004938	DAPK1	Death-associated protein kinase 1	13.5803 ↓
Hs.469658	NM_006538	BCL2L11	BCL2-like 11 (apoptosis facilitator)	5.2596 ↓
Hs.5353	NM_001230	CASP10	Caspase 10, apoptosis-related cysteine peptidase	4.0726 ↓
Hs.591834	NM_003844	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	3.967 ↓
Hs.513667	NM_003946	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	2.8887 ↓
Hs.501497	NM_001252	CD70	CD70 molecule	2.8129 ↓
Hs.87247	NM_003806	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	2.5816 ↓
Hs.710305	NM_004536	NAIP	NLR family, apoptosis inhibitory protein	2.3876 ↓
Hs.643120	NM_000875	IGF1R	Insulin-like growth factor 1 receptor	2.1732 ↓
Upregulated				
Hs.127799	NM_001165	BIRC3	Baculoviral IAP repeat-containing 3	10.851 ↑
Hs.462529	NM_003790	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	4.0981 ↑
Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	4.0907 ↑
Hs.654459	NM_001561	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	2.9875 ↑
Hs.522506	NM_021138	TRAF2	TNF receptor-associated factor 2	2.8331 ↑
Hs.9216	NM_001227	CASP7	Caspase 7, apoptosis-related cysteine peptidase	2.2033 ↑
Hs.145726	NM_001205	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	2.0258 ↑
Hs.194726	NM_004874	BAG4	BCL2-associated athanogene 4	2.0241 ↑

Genes identified as having a change in expression > 2-fold induced by the addition of PRP in comparison to 2% FBS are shown.

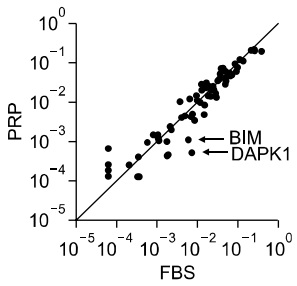

Figure 4. The results of the analysis of the expression of apoptosis-related genes affected by PRP. Total RNA was extracted from the cells cultured in the presence of 2% FBS or 2% PRP for 72 hr. The total RNA was subjected to cDNA synthesis and subsequent quantification of the mRNA expression for various apoptosis-related genes. The fold-changes in the quantified mRNA amounts compared to the average value of house-keeping genes were plotted for each gene (x-axis; cultured in 2% FBS, y-axis; cultured in 2% PRP).

analyzed. The DAPK1 mRNA level was increased 10.6-fold by serum starvation in comparison to the level in the cells cultured with 10% FBS, and the mRNA level was decreased to that of 10% FBS by the presence of 2% PRP. It is worth noting that, although the mRNA levels of DAPK1 in the cells cultured with 2% FBS and 2% PPP significantly decreased in comparison to those cultured without serum, the reductions were by 20.4% and 11.9%, respectively, which were less than that (93.6%) induced by culture in 2% PRP. The BIM mRNA levels were also drastically increased in the cells cultured in serum free medium in comparison to those cultured in the medium with 10% FBS (Figure 2C). The mRNA levels were reduced to those observed in the cells cultured with 10% FBS by the addition of 2% PRP. Again, the potential of 2% PRP to inhibit the mRNA expression of the target gene (by 87.2%) was significantly higher than that of the 2% FBS (54.1%) or 2% PPP (72.2%). Thus, PRP almost completely inhibited the expression of apoptosis-related genes induced by serum starvation.

Discussion

PRP inhibited the volume reduction of the 3D gels embedded with ccdPAs, the homogeneous preadipocytes, in comparison to the same concentration of FBS, and this was accompanied by increased cell viability in the gel. These observations prompted us to analyze the effects of PRP on the apoptosis and proliferation of the ccdPAs. The results showed that 2% PRP had a higher inhibitory effect on the apoptotic cell death of ccdPAs than 2% FBS or 2% PPP (Figure 3). A comparison between 2% PRP and 2% FBS by a gene expression profile analysis revealed that PRP downregulated 11% of the 84 representative apoptosis-related genes and upregulated 10% of the 84 representative apoptosis-related genes (Figure 4 and Table 1). The most drastically reduced genes were DAPK1, the protein product of which plays important roles in a wide range of signal transduction pathways with diverse outcomes, such as apoptosis, autoph-

agy and immune responses (Lin *et al*., 2010), and BIM, encoding one of the BH3-only proteins, which is a critical regulator of apoptosis in many cell types (Ramesh *et al*., 2009). The induction of these genes by apoptotic stimuli was almost completely prevented in the presence of PRP (Figure 5).

PRP, a concentrate of physiological cytokines, has been widely utilized as an injectable material in the clinic since the 1970s to enhance soft and hard tissue healing (Andia *et al*., 2010; Lopez-Vidriero *et al*., 2010; Redler *et al*., 2011; Yu *et al*., 2011), mainly by stimulating cell proliferation and angiogenesis in the injured tissues. PRP promotes the growth of various cells, including tissue-derived progenitor cells (Liu *et al*., 2002; Lucarelli *et al*., 2003; Doucet *et al*., 2005; Frechette *et al*., 2005; Vogel *et al*., 2006; Kakudo *et al*., 2008; Kurita *et al*., 2008; Cervelli *et al*., 2009; Chieregato *et al*., 2011), and increases the revascularization of the transplanted tissues (Bir *et al*., 2009). Based on these findings, the clinical applications of PRP have been broadened to recommend its use as an additive to tissue/cell transplantation therapies in plastic and reconstructive surgeries, and more recently in regenerative medicine. In fact, PRP has been shown to improve the fat graft survival (Abuzeni and Alexander, 2001; Sadati *et al*., 2006; Cervelli *et al*., 2009; Nakamura *et al*., 2010; Pires Fraga *et al*., 2010; Oh *et al*., 2011) and bone and periodontal regenerations *via* cell transplantation (Tobita *et al*., 2008; Chen *et al*., 2010; Yamada *et al*., 2010; Arvidson *et al*., 2011).

In order to apply PRP for clinical transplantation therapy using preadipocytes cultured *in vitro*, it is necessary to elucidate the effects of PRP on cell survival in the grafts. However, the mechanisms by which PRP increases graft survival have not been well-characterized so far. The current study showed that PRP strongly induces the proliferation of ccdPAs, preadipocytes which were previously shown to be more adipogenic than ASCs (Asada *et al*., 2011), compared with FBS at an equivalent concentration. Vogel *et al*. (2006) described that, because the addition of 2% PRP did not result in sufficient thrombocyte-clot formation to maintain a clot in the medium, a higher concentration of PRP, 3%, was evaluated for the stimulation of the MSC proliferation. In this study, to evaluate the efficacy of lower concentrations of PRP, the PRP was activated by thrombin to release cytokines (Aiba-Kojima *et al*., 2007) prior to the experiments. As a result, 2% PRP showed almost the same effects on proliferation as 10% PRP, indicating its usability as a substitute for FBS in the expansion of preadipocytes for clinical applications. Finally, 2% PRP showed anti-apoptotic activities on the preadipocytes, providing evidence that it can be used as an efficacious additive in the cell transplantation cocktail.

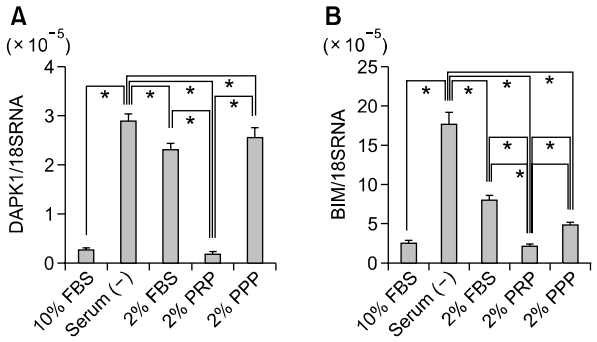

Figure 5. Serum starvation induced the expression of the DAPK1 and BIM genes, which was prevented by culture with 2% PRP. The ccdPAs were seeded and incubated for 16 hr in DMEM/HAM containing 20% FBS, and the culture medium was then replaced with medium without serum (Serum (-)), or with 2% or 10% FBS, 2% PRP, or 2% PPP, followed by incubation for an additional 72 hr. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to evaluate the mRNA expression levels of DAPK1 (A) and BIM (B). The quantification of the given genes was expressed as relative mRNA level compared with a control after normalization to 18S RNA. *$P < 0.05$.

We observed that the expression of the DAPK1 and BIM genes was substantially upregulated by serum starvation in ccdPAs. However, the addition of PRP in the growth media effectively inhibited the apoptosis and downregulated the expression of these genes. TGF-β has been shown to induce the expression of DAPK1 and BIM, and to lead to subsequent apoptosis in other cell types (Jang *et al*., 2002; Wildey *et al*., 2003; Ramjaun *et al*., 2007; Yu *et al*., 2008). PRP may therefore inhibit the TGF-β-induced apoptosis cascade(s) during serum starvation in ccdPAs. Further analyses are needed to elucidate the mechanism(s) underlying the inhibitory potential of PRP on the expression levels of the representative apoptotic genes. The gene expression analysis also showed that PRP regulated the expression levels of genes involved in TNF signaling (TNFRSF10A, TNFRSF25, TNFSF10, TNFRSF9, and TRAF2), and of the Bcl protein superfamily, with its related proteins (HRK, BNIP1, and BAG4) (Table 1). The changes in the expression of these genes may also improve the survival of ccdPAs by modulating the apoptotic stimuli, considering that TNF-α signaling plays an important role in the regulation of the adipose tissue mass (Warne, 2003).

In conclusion, PRP inhibits cell apoptosis as well as or better than FBS, and also promotes the proliferation of the ccdPAs. The gene expression analyses identified that the DAPK1 and BIM genes were the most highly downregulated apoptosis-related

genes by PRP treatment in the preadipocytes. The identified characteristics of PRP with regard to the preadipocytes have advantages including increases in the cell number and improved cell survival in the transplanted grafts. Together with our findings for the efficacies of fibrin scaffold in transplantation of ccdPAs (Aoyagi *et al*., 2011), the use of PRP for cell preparation and implantation of fat tissues and/or propagated cells may provide the graft with stable long-term survival after auto-transplantation.

Methods

Cell culture

Subcutaneous adipose tissues were obtained from healthy donors after informed consent was obtained, with approval from the ethics committee of Chiba University School of Medicine, and all studies were performed according to the guidelines of the Declaration of Helsinki. The preparation of the ceiling culture-derived proliferative adipocytes (ccdPAs) was performed as described previously (Kuroda *et al*., 2011). Dulbecco's modified Eagle's medium/F12-HAM (DMEM/HAM, Sigma-Aldrich, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) and 40 μg/ml gentamicin (GENTACIN, Schering-Plough Co., Kenilworth, NJ) was used as the culture media, unless otherwise noted in the text.

Preparation of PRP

Human PRP and PPP were prepared from healthy donors as follows; 52 ml of blood was obtained from the donors and mixed with 8 ml of Anticoagulant Citrate Dextrose Solution Formula A (ACD-A, TERUMO, Tokyo, Japan) solution, and transferred to 15 ml tubes. The tubes were centrifuged at 300 $\times$ *g* for 15 min at 20°C. The plasma and the buffy coat below the plasma were collected and transferred to new tubes. Secondary centrifugation was performed at 2000 $\times$ *g* for 15 min at 20°C. The clear supernatant (plasma) was decanted off until 6 ml was left and the middle portion of supernatant (plasma) was taken to be used as PPP. Finally, the remaining supernatant including the buffy coat was taken to be used as PRP. The platelet number of each product was automatically measured (XS 800i, sysmex Japan). The PRP utilized in this study contained 8.6 $\times$ 10^6 platelets/μl, which was approximately 7-fold concentrated from the original concentration in whole human plasma. Preparations of serum lysates containing platelet-released growth factors were essentially performed according to the method described by Aiba-Kojima *et al*. (2007). In brief, 2 U/ml of thrombin (Astellas Pharma Inc. Tokyo, Japan) was added to PRP and PPP, and the samples were agitated for 1 hr at 37°C and then incubated overnight at 4°C. Platelet bodies and any remaining fibrin were eliminated by centrifugation (2000 $\times$ *g* for 10 min), and the supernatants were obtained for the PRP and PPP. The serum samples were frozen at -20°C and thawed at 37°C before use. The growth medium was supplemented with 2 U/ml of heparin (Novo-Heparin, 5,000 units/5 ml for Injection, Mochida Pharm. Co. Tokyo, Japan).

Culture on fibrin scaffolds

Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was used as a clinically available material to generate the fibrin gel. Fibrinogen and thrombin solutions were diluted with Ringer's Solution (Fuso Pharmaceutical Industries, Osaka, Japan) containing 0.5% human serum albumin (Mitsubishi Tanabe Pharma., Tokyo, Japan). The ccdPAs were suspended at 1 $\times$ 10^7 cells/ml by the diluted fibrinogen and thrombin solution. The final concentration of fibrinogen was 4 mg/ml and the thrombin solution was used at 1 U/ml. To form fibrin clots, 50 μl of the cell-fibrinogen suspension was added to each cell culture insert (Falcon 3104; Becton Dickinson, Franklin Lakes, NJ), then shortly thereafter, 50 μl of the cell-thrombin suspension was added into the insert, mixed by pipetting, and incubated at room temperature for 2 h. The inserts with fibrin clots were put on 12 well culture plates, and culture media were added to the inserts and wells. The plates were incubated at 37°C for 12 h in a 5% CO_2 incubator, and the media were replaced by fresh media containing FBS or PRP.

LDH assay

LDH released into the culture supernatant from the FG/ccdPA was measured using the CytoTox-One Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI). A 100 μl sample of each culture supernatant was collected and added into to a 96-well plate. An equal volume of CytoTox-One Reagent was added and incubated for 10 min. Fifty μl of Stop Solution was added and the sample fluorescence was measured on a fluorescence microplate reader (SPECTRA max GEMINI XPS, Molecular Devices, Carlsbad, CA) using a wavelength of 560 nm/590 nm for excitation/emission. The original culture medium before the serum concentration was changed served as a pre-treatment control sample and the control value was subtracted from the value obtained after incubation with the medium containing different concentrations of serum.

Cell proliferation assay

The cell proliferation was examined using the CyQUANT^R Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA). Cells were seeded into 96 well plates at a density of 2 $\times$ 10^3 cells per well with DMEM/HAM/20% FBS. After 24 h, the culture medium was removed and changed to fresh DMEM/HAM without serum, or with FBS, PRP or PPP. After 3 days, the microplates were gently inverted and blotted onto paper towels to remove the medium from the wells. The microplates were then frozen and stored at -80°C and thawed at room temperature prior to analysis. CyQUANT GR dye/cell-lysis buffer was added to each well. Cells were incubated at room temperature for 5 min and the sample fluorescence was measured on a fluorescence microplate reader (SPECTRA max GEMINI XPS, Molecular Devices) using wavelength of 480 nm/520 nm for excitation/emission.

Induction of apoptosis, and the annexin V binding and caspase 3 activity assays

The cells were seeded into 10 cm dishes at a density of 1×10^6 cells per well with DMEM/HAM/20%FBS. After 24 h, the culture medium was removed and changed to fresh DMEM/HAM with 2% FBS, 2% PRP or 2%PPP. After 2 h, apoptosis was induced by the addition of 100 ng/ml TNF-α (Peprotech, Rocky Hill, NJ) and 100 μg/ml cycloheximide (CHX, Sigma-Aldrich). After 3 h, the culture supernatant was collected, and the cells were detached by TrypZean treatment. The detached cells were suspended in the collected culture supernatant. Subsequently, the cells were stained with Annexin V-FITC using a Tali™ Apoptosis kit (Life Technologies). Stained cells were analyzed by a Tali™ Image Based Cytometer (Life technologies). Cell lysates at the concentration of 1×10^4 cells/μl were prepared from the cells treated to induce apoptosis, and the caspase 3 activity levels were measured by a caspase-3/CPP32 Fluorometric Assay Kit (Biovision, Mountain View, CA). To examine the phosphorylation status of ERK1/2, the cells were scraped off at each time point in PBS and washed. The cells were pelleted and lysed by RIPA buffer (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and the protein concentration was determined by Quant-iT Protein Assay Kit (Life technologies), and 5 μg of protein were analyzed by Western blotting using anti-ERK1 and anti-ERK1/2 (pT202/pY204) as primary antibodies (BD Biosciences, Franklin Lakes, NJ). Mouse TrueBlot ULTRA HRP-conjugated Anti-Mouse IgG (eBioscience, Inc. San Diego, CA) was used as a secondary antibody, and the signals were detected by SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.) with LAS1000 apparatus (FUJI film, Tokyo, Japan). ERK signals were normalized using Actin signals detected by Anti-Actin monoclonal antibody (clone AC-40, Sigma-Aldrich).

Gene expression analysis

Cells (2.5×10^5 cells) were seeded into 10 cm dish with DMEM/HAM/20% FBS and cultured for 16 h. The culture medium was changed to fresh DMEM/HAM supplemented with 2% FBS or 2% PRP, and the cells were further incubated for 72 h. The total RNA from cultured cells was extracted using a RT^2 RNA Isolation Kit (SA Bioscience, Frederick, MD). Complementary DNA was generated from 1 μg of total RNA using the RT^2 First Strand Kit. An Apoptosis Reverse Transcriptase RT^2 profiler PCR array and RT^2 Real-Time SYBR Green/ROX PCR Mix (SA Bioscience, Frederick, MD) were used to identify the genes affected by PRP according to manufacturer's instruction. The data were analyzed by web-based data analysis software provided by the manufacturer. The probe and primer sets used to quantify the mRNA for the DAPK1 and BIM genes were purchased from Applied Biosystems (Life Technologies). The quantification of given genes was expressed as the relative mRNA level compared with a control after normalization to 18S RNA. All the real-time PCR were performed using an ABI 7500 real-time PCR apparatus.

Statistical analysis

The data are presented as the means $\pm$ S.D. Statistical comparisons were made by Student's *t*-test or an ANOVA followed by the post-hoc Tukey test using the SPSS software program. In all cases, *P*-values < 0.05 were considered to be significant.

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