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Platelet-Rich Plasma Increases Growth And Motility Of Adipose Tissue-Derived Mesenchymal Stem Cells And Controls Adipocyte Secretory Function

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ABSTRACT

Adipose tissue-derived mesenchymal stem cells (Ad-MSC) and platelet derivatives have been used alone or in combination to achieve regeneration of injured tissues. We have tested the effect of platelet-rich plasma (PRP) on Ad-MSC and adipocyte function. PRP increased Ad-MSC viability, proliferation rate and G1-S cell cycle progression, by at least 7-, 2-, and 2.2-fold, respectively, and reduced caspase 3 cleavage. Higher PRP concentrations or PRPs derived from individuals with higher platelet counts were more effective in increasing Ad-MSC growth. PRP also accelerated cell migration by at least 1.5-fold. However, PRP did not significantly affect mature adipocyte viability, differentiation and expression levels of PPAR-γ and AP-2 mRNAs, while it increased leptin production by 3.5-fold. Interestingly, PRP treatment of mature adipocytes also enhanced the release of Interleukin (IL)-6, IL-8, IL-10, Interferon-γ, and Vascular Endothelial Growth Factor. Thus, data are consistent with a stimulatory effect of platelet derivatives on Ad-MSC growth and motility. Moreover, PRP did not reduce mature adipocyte survival and increased the release of pro-angiogenic factors, which may facilitate tissue regeneration processes. J. Cell. Biochem. 116: 2408–2418, 2015.

KEY WORDS: GROWTH FACTORS; TISSUE REGENERATION; ADIPOSE TISSUE; CYTOKINES

The use of platelet derivatives represents a novel approach in tissue regeneration [Burnouf et al., 2013]. Although the mechanisms involved are still poorly understood, the relative ease and safety of application of platelet products in clinical practice and the potential beneficial outcomes, including regeneration of bone and soft tissues, reduction of bleeding, and acceleration or promotion of wound healing, hold promise for new therapeutic approaches [Nikolidakis and Jansen, 2008; Lacci and Dardik, 2010; Burnouf et al., 2013].

The rationale for the use of platelet products is mostly based on the production and release of multiple growth factors upon platelet activation. Primary factors associated with platelets include Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor β (TGF-β), which have been mostly involved in cell proliferation, chemotaxis, and extracellular matrix production/angiogenesis [Lubkowska et al., 2012; Burnouf et al., 2013]. Other growth factors discharged from the platelets are Fibroblast Growth Factors (FGF) 1 and 2 and Vascular Endothelial Growth Factor

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Vittoria D'Esposito and Federica Passaretti equally contributed to this work.

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(VEGF) which play critical roles in the hemostasis, proliferative, and remodeling phases of wound healing [Intini, 2009; Lacci and Dardik, 2010; Demidova-Rice et al., 2012; Lubkowska et al., 2012]. More than 95% of the pre-synthesized growth factors are secreted within 1 h from the beginning of the clotting process. After the initial burst, the platelets secrete additional growth factors for the remaining 7 days of their life span [Marx, 2004]. Platelet degranulation also leads to the release of cytokines and chemokines, further contributing to the healing process [Demidova-Rice et al., 2012; Passaretti et al., 2014].

Several technical procedures have been developed to obtain platelet concentrates [Dohan Ehrenfest et al., 2009]. Different procedures lead to variable yield of platelets and may contain different cellular components [Prakash and Thakur, 2011; Cieslik-Bielecka et al., 2012]. Consistently, they also differ for the qualitative and quantitative release of growth factors, cytokines and chemokines and may find different indications [Galliera et al., 2012; Lubkowska et al., 2012; Passaretti et al., 2014]. Attention has also been given to the clinical utilization of individual growth factors. However, the overall experience has not been satisfactory, most likely since wound healing and tissue repair are outcomes of an intricate network of circulating and tissue elements. Combination of multiple growth factors, timing of their release, and cell-specific response to individual growth factors are indeed essential requirements for a successful wound healing [Giacco et al., 2006].

Auto-transplantation of adipose tissue is commonly used for the treatment of tissue defects in plastic and reconstructive surgery. The reduced survival of the transplanted adipose tissue remains an unsolved issue. This is due, at least in part, to accelerated apoptosis of the implanted pre-adipocytes. Several reports have indicated that application of Platelet-Rich Plasma (PRP) may improve the outcome of adipose tissue transplantation [Cervelli et al., 2009; Fukaya et al., 2012]. The molecular mechanisms may possibly involve interactions of PRP factors with either adipocytes and mesenchymal stem cells, which are embedded within adipose tissue (Ad-MSC) [Liu et al., 2008]. MSCs are multipotent, non hematopoietic stem cells that are typically obtained from bone marrow but can also be isolated from several other tissues such as umbilical cord and adipose tissue [de Girolamo et al., 2013]. Human Ad-MSCs are attractive candidates for clinical use because of their ease of isolation, extensive proliferation and differentiation capacity, and hypoimmunogenic nature. Ad-MSCs display the ability to differentiate into multiple mesoderm-derived cells, such as adipocytes, osteocytes, and chondrocytes, but they may also give rise to cells of nonmesodermal origin, such as hepatocyte-like and neuronal-like cells. The multilineage capacity of Ad-MSC offers the potential to repair, maintain or enhance regeneration of various tissues [Kocaoemer et al., 2007; Phinney and Prockop, 2007; Schäffler and Büchler, 2007; Tran and Kahn, 2010]. However, the poor viability of Ad-MSCs at the transplanted site often decreases their therapeutic potential [Nakamura et al., 2013]. Thus, it is important to improve Ad-MSC survival and enhance their biological functions.

The combined use of PRP and Ad-MSC offers the advantage that they both could be autologous products, prepared from the patient's own tissues, thereby eliminating concerns about immunogenic reactions and disease transmission. Nevertheless, in some cases, preparations from donors could be needed [Everts et al., 2007; Liu et al., 2008].

Despite the large utilization of platelet derivatives as therapeutic tools in conditions requiring tissue repair, little has been reported about the molecular mechanisms elicited by PRP on adipose tissue function. In this paper, we have described that PRP affects Ad-MSC growth and migration without interfering with their ability to differentiate into mature adipocytes. Moreover, the exposure to PRP sustains adipocyte viability and increases the production of specific factors, thereby contributing to the induction of tissue repair processes.

MATERIALS AND METHODS

MATERIALS

Media, sera, and antibiotics for cell culture were from Lonza (Lonza Group Ltd, Basel, Switzerland). Antibodies against phospho-Ser_{473}PKB/Akt1, ERK, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Phospho-Thr_{202}/Tyr_{204}ERK, antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-active + pro Caspase 3 antibody was purchased from Abcam (Cambridge, UK), PKB/Akt antibody was from Millipore (Millipore, Billerica, MA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) reagents from Bio-Rad (Bio-Rad, Hercules, CA). All the other chemicals were from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO).

SUBJECT RECRUITMENT AND PLATELET-RICH PLASMA (PRP) PREPARATION

Ten subjects undergoing biliary surgery (M/F: 4/6; age 24–40) were enrolled in the study. All were non-smokers, non-obese (BMI range: 20.4–28.3) and with a platelet count $>180.000/mm^3$. None of them were under any medication for the last 21 days. Informed consent was obtained from every subject before the surgical procedure. This procedure was approved by the ethical committee of the University of Naples.

Blood was drawn from each subject and was collected in a Vacutainer tube (Vacutainer; Becton Dickinson, East Rutherford, NJ) containing 10% trisodium citrate anticoagulant solution for the preparation of Platelet-Rich plasma (PRP). Tubes were centrifuged at 350 g for 15 min. The upper fraction containing platelet-poor plasma (PPP) was set apart and the preparation procedure for PRP were performed as previously described [Passaretti et al., 2014].

For platelet gel preparations, autologous thrombin (0.1 NIH unit/ml final concentration) and calcium gluconate (10 mg/ml final concentration) were added to PRP for 5 min at room temperature to allow clot formation [Giacco et al., 2006].

HUMAN ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELL CULTURE, GROWTH, AND VIABILITY

Human adipose tissue biopsies were digested with collagenase and Mesenchymal Stem Cells (Ad-MSC) were isolated and differentiated as previously reported [D'Esposito et al., 2012]. For growth evaluation, Ad-MSCs (5×10^4 cells) were seeded in 6-well culture plates in a complete medium. The following day, the cells were

starved in serum-free DMEM (Dulbecco's modified Eagle's Medium) -F12 (1:1) 0.25% BSA for 16 h and incubated with PRP gel, obtained as described above, for different times. Cell count was performed either by Bürker chamber and with the TC10^{TM} Automated Cell Counter (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Sulforhodamine assay was used for cell viability determination [Chiba et al., 1998]; cells were fixed with 10% trichloroacetic acid for at least 2 h at 4 C and then washed five times with distilled and de-ionized water. After air drying, sulforhodamine was added to the cells and incubated for 30 min. Cells were then washed with 1% acetic acid five times. After air drying, 10 mmol/l Tris solution (pH 7.5) was added to dissolve the bound dye. Cell viability was assessed by optical density (OD) determination at 510 nm using a microplate reader. Three replicate wells were used for each data point.

CELL CYCLE ANALYSIS

Ad-MSCs were seeded in 10 cm^2 culture plates in a complete medium. The following day, the cells were starved in serum-free DMEM F12 (1:1) 0.25% BSA for 16 h and incubated with PRP gel obtained as described above. Bromodeoxyuridine (BrdU)/Propidium Iodide (PI) analysis was performed as previously reported [Ma et al., 2013]. Briefly, cells were labeled with 10 μM BrdU for 30 min and fixed over-night in ice-cold 70% ethanol at −20 C. Cells were then washed once with PBS and incubated for 15 min at room temperature with 2N HCl. Next, cells were washed with PBT (0.5% BSA, 0.1% Tween20 in PBS) and resuspended in PBT containing anti-BrdU antibody (1:40, Dako, Carpinteria CA). After 30 min, cells were washed twice with PBT and then resuspended in PBT containing Alexa488 anti-mouse (1:100, Life Technologies, Carlsbad, CA) in the dark for 30 min. Cells were washed twice with PBS, resuspended in Propidium Iodide (PI) 0.015 M (Sigma–Aldrich) in PBS for 20 min and analyzed for the emission in FL1 and FL3 channels. The samples were acquired by a BD LSRFortessa (BD Biosciences, San Jose, CA) and analyzed using BD FACSDiva Software.

CELL MIGRATION

Cell migration was performed using 8 μm pore polycarbonate membranes (Costar, Cambridge, MA). Ad-MSCs were loaded at 50,000 cells per insert (upper chamber) and PRP gel was added to the lower chamber in DMEM F12 (1:1) 0.25% BSA. The cells were allowed to migrate into the lower chamber at 37 C in a 5% CO_2 atmosphere saturated with H_2O for 24 h in presence of mitomycin C (10 mg/ml; Sigma–Aldrich). At the end of incubation, cells that had migrated to the lower side of the filter were fixed with 11% glutaraldehyde for 15 min at room temperature, washed three times with PBS, and stained with 0.1% crystal violet-20% methanol for 20 min at room temperature. After three PBS washes and complete drying at room temperature, the crystal violet was solubilized by immersing the filters in 10% acetic acid. The concentration of the solubilized crystal violet was evaluated as absorbance at 540 nm.

ADIPOCYTE DIFFERENTIATION MARKERS AND REAL-TIME RT-PCR ANALYSIS

Adipocyte differentiation was assessed by the analysis of real-time RT-PCR of adipocyte-specific (aP2 or PPARγ) mRNAs (see below) and by lipid accumulation using Oil Red O staining, as described by Ramirez-Zacarías et al. [1992]. Total RNA was isolated from either undifferentiated Ad-MSC and differentiated adipocytes, after removal of PRP gels, by using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. For real-time RT-PCR analysis, 1 μg cell RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA). PCR were analyzed using SYBR Green mix (Bio-Rad, Hercules, CA). Reactions were performed using Platinum SYBR Green Quantitative PCR Super-UDG using an iCycler IQ multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All reactions were performed in triplicate and β-actin was used as an internal standard. Primer sequences are described in Table I.

IMMUNOBLOT PROCEDURE

Total cell lysates were obtained and separated by SDS–PAGE as previously described [Alberobello et al., 2010]. Briefly, cells were solubilized for 20 min at 4 C with lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride,10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4, and 1% (v/v) Triton X-100. Lysates were clarified by centrifugation at 12,000 g for 20 min at 4 C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted on Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked for 1 h in TBS (10 mM Tris-HCl, pH 7.4, and 140 mM NaCl) containing 3% (w/v) bovine serum albumin and then incubated with the indicated antibodies. Detection of blotted proteins was performed by ECL according to the manufacturer's instruction. Densitometric analysis was performed using Image Lab software (Bio-Rad, Hercules, CA).

CONDITIONED MEDIA COLLECTION

Adipocytes were incubated with PRP gels, obtained as described above, for 24 h. Next, PRP gels were removed and the cells were starved in serum-free DMEM F12 (1:1) 0.25% BSA. As control, PRP gels, without cells, were maintained in serum-free DMEM F12 (1:1) 0.25% BSA. After 24 h media were collected and centrifuged at 14,000 g to remove cellular debris and analyzed for cytokines and growth factor content, as described below.

DETERMINATION OF CYTOKINE AND GROWTH FACTOR RELEASE

PRP and adipocyte conditioned media were screened for the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, TNF-α, bFGF, PDGF, VEGF using the Bioplex multiplex Human Cytokine and Growth factor assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

TABLE I. Primer Sequences Used in Real-Time RT-PCR Analysis

PPARγ	Forward 5′-GACCACTCCCACTCCTTTGA -3′
	Reverse 5′ -GATGCAGGCTCCACTTTGAT - 3′
AP2	Forward 5′-TCACAGCACCCTCCTGAAAACTGC- 3′
	Reverse 5′-TTGGCCATGCCAGCCACTTTCC- 3′
Leptin	Forward 5′-CACACACGCAGTCAGTCTC- 3′
	Reverse 5′-GAGGTTCTCCAGGTCGTTG- 3′
β-actin	Forward 5′-GCGTGACATCAAAGAGAAG- 3′
	Reverse 5′-ACTGTGTTGGCATAGAGG- 3′

STATISTICAL ANALYSIS

Data were analyzed with Statview software (Abacus Concepts Piscataway, NJ). A one-factor analysis of variance (ANOVA) was used to analyze PRP effect. Any significant ANOVA were further analyzed by Student- Neuman- Keuls post hoc test to determine the specificity of the effect. *P* values of less than 0.05 were considered statistically significant.

RESULTS

PRP PROMOTES AD-MSC SURVIVAL, GROWTH, AND MIGRATION

We have first analyzed the impact of PRP-released factors on human Mesenchymal Stem Cells isolated from stromal-vascular fraction of subcutaneous adipose tissue biopsies (Ad-MSC). PRP (platelet count 300,000–400,000/μl) and PPP (<200,000 plts/μl) were activated with thrombin and applied onto cultured human Ad-MSCs as PRP or PPP gel, respectively (5% or 20% vol/vol in cell colture medium). As assessed by sulforhodamine assay, Ad-MSC viability was strongly increased in presence of 5% or 20% PRP gel, compared to that measured in serum deprivation and was about 3- and 4-fold higher, respectively, compared to cells cultured in 10% FBS (Fig. 1a). As expected, 5% PPP induced an increase of Ad-MSC growth which was comparable to that measured in 10% FBS, while 20% PPP increased it by 2.4-fold. However, the effect of PPP on cell viability was significantly lower than that achieved with similar concentration of PRP (Fig. 1a).

To further investigate whether PRP growth promoting action was dependent on blood platelet count, Ad-MSCs derived from one subject were incubated with PRP gels obtained from grouped subjects according to concentration of blood platelets; those with "low" (200,000–300,000/μl) platelet count (n = 5) and those with "high" (400,000–500,000/μl) platelet count (n = 5). Ad-MSC growth was significantly higher upon incubation with the PRP obtained from individuals with a higher concentration of platelets (Fig. 1b) and reached the confluency after 48 h from seeding. In addition, PRP gels elicited cell growth when applied onto cells isolated either from the same blood donor (autologous PRP) or from other individuals (homologous PRP) (data not shown).

Moreover, BrdU/PI staining revealed that both 5% and 20% PRP gel addition increased the amount of Ad-MSCs in S-phase, compared to cells cultured in serum-free medium without PRP (Fig. 1c). PRP gel reduced the number of cells in G1 phase without affecting G2-M and sub G1 phases (Supplementary Online Table).

To investigate whether PRP could also ameliorate Ad-MSC migration, cells were placed in the upper chamber of a transwell system, while the lower chamber was filled up with PRP-gel in serum-free medium. Cells that migrated across the filter were detected and quantified. 5% and 20% PRP increased Ad-MSC migration by 1.5- and up to 2-fold compared to serum-free medium (Fig.2a–b).

We next tested whether PRP could activate intracellular signaling pathways involved in cell growth and apoptosis. To this aim, Ad-MSC were incubated with PRP gel for 12 h. Western blot analysis with phospho-specific antibodies revealed that 5% and 20% PRP increased PKB/AKT, ERK and reduced Caspase 3 cleavage compared to the control untreated cells (Fig. 2c and d).

PRP DOES NOT INTERFERE WITH AD-MSC ADIPOGENIC DIFFERENTIATION, SURVIVAL, AND FUNCTION

Ad-MSCs readily differentiate into cells of the adipocyte lineage and retain differentiation potential through multiple passages [Schäffler and Büchler, 2007]. In order to evaluate whether PRP treatment may interfere with adipocyte differentiation, Ad-MSC were incubated with PRP along with the induction of the differentiation procedure as described in Materials and Methods. Adipogenesis was assessed by analysis of lipid accumulation using oil red O staining (Fig. 3a) and by the expression of adipocyte-specific genes (aP2 and Peroxisome Proliferator-Activated Receptor γ –PPARγ) (Fig. 3b). Ad-MSC differentiation in presence of 20%PRP was comparable to that observed for cells differentiated with the standard procedures (Fig. 3a and b), with a slight, not significant, increase of cell size.

In addition, we have tested the effect of PRP on mature adipocytes. Sulforhodamine assay revealed that 20% PRP gels lightly increased adipocyte viability, although this effect was not significant (Fig. 3c). Interestingly, leptin expression was 3-fold higher in differentiated adipocytes exposed to PRP (Fig. 3d).

RELEASE OF CYTOKINES/CHEMOKINES AND GROWTH FACTORS BY HUMAN ADIPOCYTES UPON PRP STIMULATION

As previously reported, PRP released a variety of cytokines/chemokines and growth factors [Passaretti et al., 2014]. Here, we have shown that PRP secreted IL-4, IL-8, CCL5/RANTES, and PDGF in a concentration dependent fashion (Table II). IL-6 and INF-γ were found only in conditioned media from 20% PRP. No statistically significant differences were observed for the amount of VEGF between 5% and 20% PRP medium (Table II). We have therefore investigated whether PRP may affect the ability of human adipocytes to release cytokines/chemokines and growth factors. As expected, several inflammatory cytokines and growth factors were detected in the medium of untreated adipocytes. Interestingly, the amount of IL-6, IL-8, IL-10, IFN-γ, and VEGF was significantly increased in PRP-treated adipocytes. However, while the increase of IFN-γ and VEGF levels was consistent with an additive release by PRP and adipocytes separately, the increase of IL-6, IL-8, and IL-10 was likely due to the PRP stimulation of release by adipocytes. Moreover, the concentrations of IL-4, PDGF, and CCL5/RANTES were significantly reduced in PRP-treated adipocyte medium, compared to PRP alone, suggesting a consumption of those platelet-released factors by the adipocytes (Fig. 4).

DISCUSSION

Platelet derivatives are widely used in regenerative medicine [Nikolidakis and Jansen, 2008; Lacci and Dardik, 2010; Burnouf et al., 2013]. For instance, the simultaneous application of fat

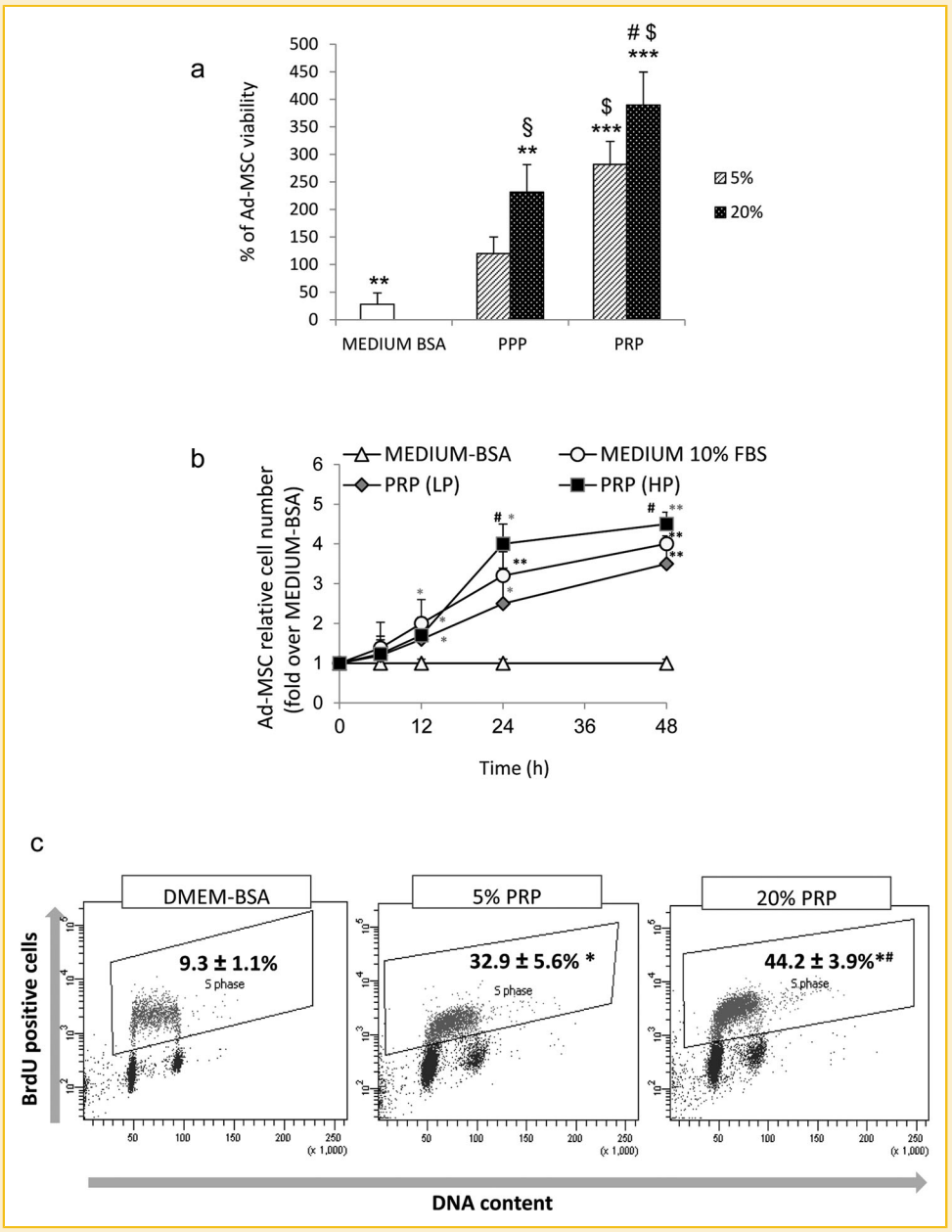

Fig. 1. Effect of PRP on Ad-MSC survival, growth and cell cycle. a) Ad-MSCs isolated from adipose tissue biopsies (n = 5) have been seeded in 96-well culture plates (3,000 cells/well). The following day the cells have been serum-starved for 18 h and then incubated with PRP or PPP gel (5% or 20% vol/vol in DMEM F12 1:1) for 48 h without serum supplementation (MEDIUM BSA). Cell viability has been assessed by sulforhodamine assay as described in Materials and Methods and the results reported as percentage of viable cells compared to cells in DMEM F12 10% FBS, considered as 100% viable cells. * denotes statistically significant values over basal, considered as cells kept in 10% FBS medium (** $P < 0.01$; *** $P < 0.001$). # denotes statistically significant difference of 20% vs. 5% PRP (# $P < 0.05$). § denotes statistically significant difference of 20% vs. 5% PPP (§ $P < 0.05$). $ denotes statistically significant difference of PRP vs PPP ($ $P < 0.05$) b) PRP gel (20% vol/vol in DMEM F12 1:1) obtained from donors with different hematic platelet counts (Low Platelet – LP:2-3 $\times 10^5$ platelets/μl; High Platelet – HP:4-5 $\times 10^5$ platelets/μl) were directly applied onto the culture plate containing serum-starved Ad-MSCs for 6, 12, 24, and 48h. As control, Ad-MSCs have been incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% FBS (MEDIUM 10% FBS). Then, cells have been counted as described in Materials and Methods and the results have been reported as cell number relative to cell count in MEDIUM BSA. * denotes statistically significant values over basal (* $P < 0.05$; ** $P < 0.01$). # denotes statistically significant differences of HP-PRP vs. LP- PRP (# p < 0.05). c) PRP gel (5% or 20% vol/vol in DMEM F12 1:1) has been added to serum-starved Ad-MSCs for 48 h. Cells have been pulse-labeled with BrdU for 30 min. FACS analysis of samples stained for BrdU and for propidium iodide, to quantify the amount of DNA, was performed. Numbers represent the percentage of BrdU positive cells ± SD. * denotes statistically significant values over DMEM-BSA (* $P < 0.05$). # denotes statistically significant differences of 20% vs. 5% PRP (# $P < 0.05$).

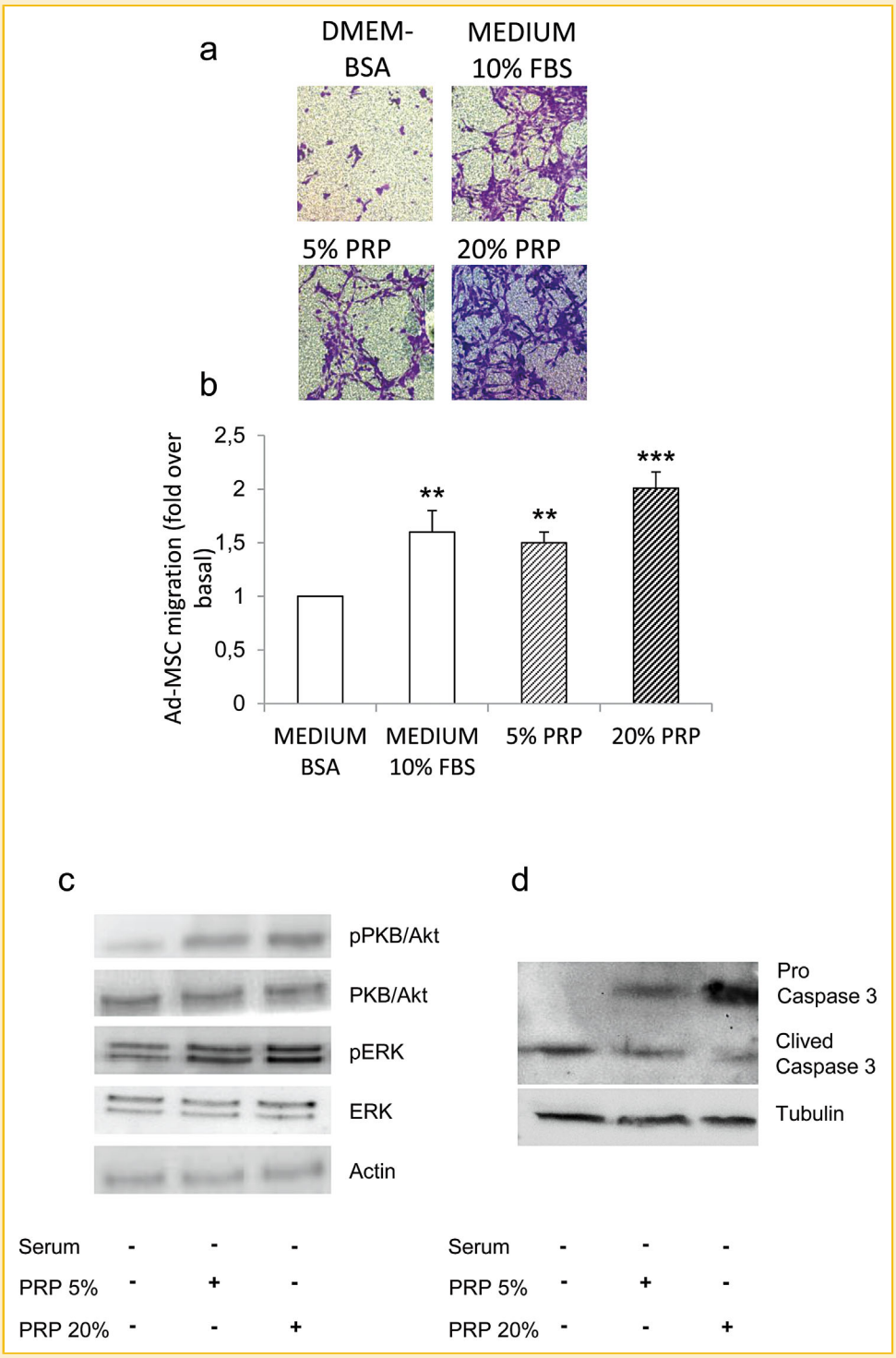

Fig. 2. Effect of PRP on Ad-MSC migration and intracellular pathway activation. Ad-MSCs have been serum-starved for 18 h and then seeded on the polycarbonate membrane in the upper compartment of the transwell in presence of 10 mg/ml mitomycin C , whereas PRP gel (5% and 20% vol/vol in DMEM F12 1:1) has been added to the lower compartment in presence of DMEM F12 (1:1) without serum supplementation. As control, DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS) has been added to the lower compartment. Upon 24 h, migratory cells on the bottom of the polycarbonate membrane were stained (a) and quantified at OD 540 nm after extraction (b). Asterisks denote statistically significant values over basal (** $P < 0.01$; *** $P < 0.001$). c–d) Ad-MSCs were exposed to PRP (5% and 20% vol/vol in DMEM F12 1:1) for 24 h and then solubilized as described in Materials and Methods. Cell lysates (50 μg protein/sample) were blotted with phospho- Ser_{473}PKB/Akt, phospho-Thr_{202}/Tyr_{204}ERK and active + pro Caspase 3 antibodies and then reblotted with anti-PKB/Akt and anti-ERK antibodies. To ensure the equal protein transfer, membranes were blotted with actin (c) or tubulin (d) antibodies. The filters were revealed by ECL and autoradiography. The autoradiographs shown are representative of four independent experiments.

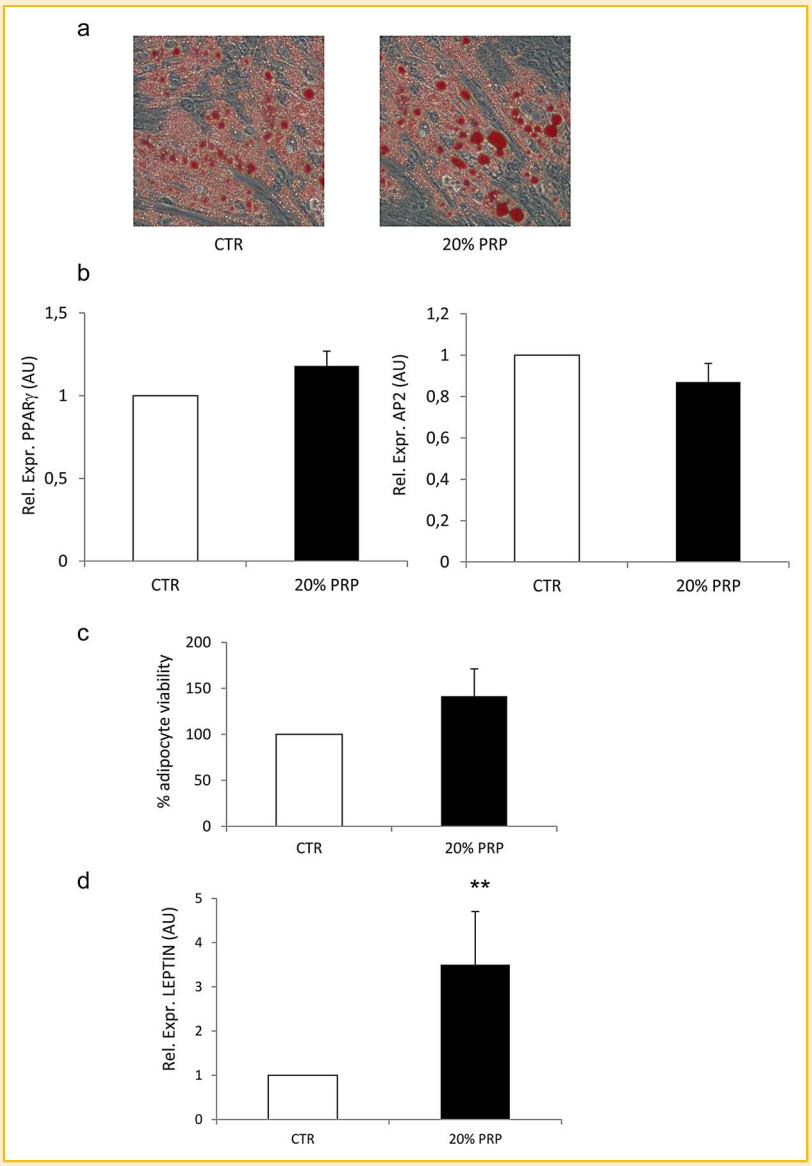

Fig. 3. Effect of PRP on adipocyte differentiation, viability and function. Ad-MSCs have been differentiated in adipocytes as described in Materials and Methods, in presence or absence of PRP gel (20% vol/vol in DMEM F12 1:1). a) Lipid accumulation has been observed by Oil Red O staining microscopically. mRNA levels of PPARγ, AP2 (b), and Leptin (d) were determined by real-time RT-PCR analysis on RNA preparations obtained from adipocytes after complete removal of PRP gels. Data have been normalized on β-actin as internal standard. Bars show the mRNA levels in these cells relative to those in Ad-MSCs differentiated without 20% PRP gel addition. c) Adipocyte viability has been assessed by sulforhodamine assay and the results reported as percentage of viable cells compared to cells differentiated without 20% PRP gel addition, considered as 100% viable cells.

explants and PRP has hold a great deal of promise to ameliorate "lipofilling" procedures and the outcome of fat engraftment [Nakamura et al., 2010; Kùlle et al., 2013]. Indeed, one of the main limitations of adipose tissue transfer is the rapid loss of fat at the site of engraftment [Nakamura et al., 2010]. This is possibly due to several factors: i) very low capability of adipose tissue to engraft and to repopulate at the site of implantation; ii) reduced life span of terminally differentiated mature adipocytes; iii) reduced blood supply mainly due to insufficient tissue-driven angiogenesis.

Here, we have tested the effect of PRP-released factors on Ad-MSCs and on mature adipocytes in cultured models. PRP-released factors had an incremental effect on Ad-MSCs, since they improved cell viability, induced S-phase and increased cell number. This is consistent with previous reports indicating a positive effect of PRP on growth of mesenchymal stem cells, either from adipose tissue [Kocaoemer et al., 2007; Cervelli et al., 2012] and from bone marrow [Murphy et al., 2012], as well as of other cultured cell types [Lucarelli et al., 2003; Giacco et al., 2006; Kakudo et al., 2008; Gassling et al., 2009; Passaretti et al., 2014]. The molecular mechanisms responsible

TABLE II. Cytokines and Growth Factors Released by PRP.PRP Gel (5% or 20% vol/vol in DMEM F12 1:1) Were Incubated With Serum-Free DMEM-F12 (1:1). After 24 h, the Media Were Collected and Tested by Using the Bioplex MultiplexHuman Cytokine and Growth Factor Assay Kit

Bio-Plex Panel	5%PRP CM (pg/ml)	20%PRP CM (pg/ml)
IL-2	ND	ND
IL-4	0.30 ± 0.08	$1.18 \pm 0.1^{*}$
IL-6	ND	7.57 ± 0.9
IL-8	149.65 ± 60.8	$450.67 \pm 25.73^{*}$
IL-10	ND	ND
MIP-1a	ND	ND
MIP-1b	ND	ND
INF-γ	ND	152.02 ± 75.62
RANTES/CCL5	1207.21 ± 65.6	$2819.84 \pm 147.6^{**}$
TNFα	ND	ND
GM-CSF	ND	ND
PDGF	2092.35 ± 64.07	$8254.66 \pm 361.72^{***}$
VEGF	90.55 ± 2.51	120.23 ± 17.32
bFGF	ND	ND

Asterisks denote statistically significant values (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

for increased cell growth likely involve PKB/Akt and ERK (Fig. 2) activation by platelet released growth factors and the potential regulation of apoptosis-related genes, which may lead to caspase 3 cleavage, as also recently described [Cervelli et al., 2012; Fukaya et al., 2012].

Growth effect on Ad-MSC is not limited to the exposure to PRP obtained from blood of the same adipose tissue donor (autologous PRP), but is also elicited by PRPs obtained from different donors (homologous PRP) and the difference observed on cell growth induced by autologous and homologous PRP is not statistically significant (data not shown). Moreover, Ad-MSC growth is elicited at higher levels by PRP compared to PPP preparations [Giacco et al., 2006; Kakudo et al., 2008; Fukaya et al., 2012] and by PRP obtained from donors with higher platelet count. Thus, as also previously suggested [Lucarelli et al., 2003], the concentration of platelet factors may be crucial in inducing cell proliferation. Indeed, Murphy et al. [2012] have recently described that PRP obtained by umbilical cord (uc-PRP) is more potent than that obtained by peripheral blood in inducing BM-MSC growth. This is possibly due to the release of higher concentrations of specific growth factors by uc-PRP. Nevertheless, growth effect is most likely cell-specific, since different types of platelet preparations release different amount of growth factors and cytokines/chemokines [Passaretti et al., 2014]. In the case of Ad-MSC, and similar as in human skin fibroblasts [Giacco et al., 2006], we found that PDGF signaling was sufficient to induce cell proliferation (data not shown).

We have now provided further evidence that PRP can exert a chemo-attractant action on Ad-MSCs. Increased motility could also be driven by PKB/Akt activation [Bulj et al., 2013]. Other mechanisms however could not be excluded. One might argue that PRP increases cellularity at the site of implant, both by inducing proliferation and recruiting more Ad-MSC. The higher amount of precursor cells may then lead to increased tissue formation, thereby contributing to improve the outcome of fat transplantation.

Thus, PRP treatment of adipose tissue may facilitate the recruitment of Ad-MSCs and induce their proliferation, without reducing the survival of mature adipocytes. The combination of these effects supports the hypothesis of a beneficial action of PRP products in adipose tissue regeneration and filling procedures.

Based on morphological data and on expression of specific markers, PRP-treated Ad-MSCs retain their ability to differentiate into adipocytes, as well as into osteogenic lineage [data not shown; Cervelli et al., 2012; Tavakolinejad et al., 2014], at least in cultured models. Nevertheless, as for cell proliferation, also for adipocyte differentiation, the choice of the platelet preparation is of crucial importance. For instance, Koellensperger and coworkers have recently described that human serum from PRP reduced the adipogenic differentiation of Ad-MSCs. Instead, data obtained from our group and others [Kocaoemer et al., 2007; Cervelli et al., 2012] suggested that the adipose tissue obtained in the presence of PRP preserved its architecture and its functional features. In addition, Cervelli and coworkers have demonstrated that PRP amplified insulin-dependent adipogenic differentiation of Ad-MSCs. Interestingly, however, we also detected an increase of leptin in PRP-treated differentiated adipocytes and increased levels of VEGF, IL-6, IL-8, IL-10, and IFN-γ. In particular, the increase of IL-6, IL-8, and IL-10 levels are more than additive and likely results from PRP stimulation of adipocyte release. On the other end, PDGF, IL-4, and CCL5/RANTES, which are robustly produced by platelets, were reduced in the medium of PRP-treated adipocytes. Thus, it could be hypothesized that PRP-released factors (i.e., PDGF) are taken and consumed by adipocytes, which in turn release more pro-angiogenic factors (i.e., VEGF and IL-6), thereby facilitating new vessel formation and further contributing to the stabilization of the transplanted fat. Alternatively, a bi-directional cross talk could be envisioned, by which adipocytes enhance degradation or inhibit production of platelet factors, although the latter appears as a less likely possibility.

Platelet secretome analysis has released a very large amount of proteins, which may independently ad coordinately act on processes involved in wound healing and tissue repair [Intini, 2009; Lacci and Dardik, 2010; Demidova-Rice et al., 2012; Lubkowska et al., 2012]. Different procedures of platelet preparations may vary in the abundance of growth factors and cytokines [Horn et al., 2010; Passaretti et al., 2014], and this may be taken into account for the variability of the outcomes of platelet-based therapies [Prakash and Thakur, 2011]. The use of individual factors, such as PDGF for example, has proven largely unsuccessful [Park et al., 2014]. More recently, peptides derived from human PRP have been isolated and tested for promoting cutaneous wound healing in animal models [Demidova-Rice et al., 2012].

We have now provided evidence that PRP may elicit chemo-attractant and proliferative effects on Ad-MSC, without interfering with adipocyte differentiation. PRP-treated adipocytes may then become more potent in secreting pro-angiogenic factors, including leptin and VEGF, and ameliorate the outcome of autologous fat implants. More studies are needed to elucidate individual factors and specific pathways involved in the intricate cross-talk among cell types responsible for wound healing and tissue regeneration.

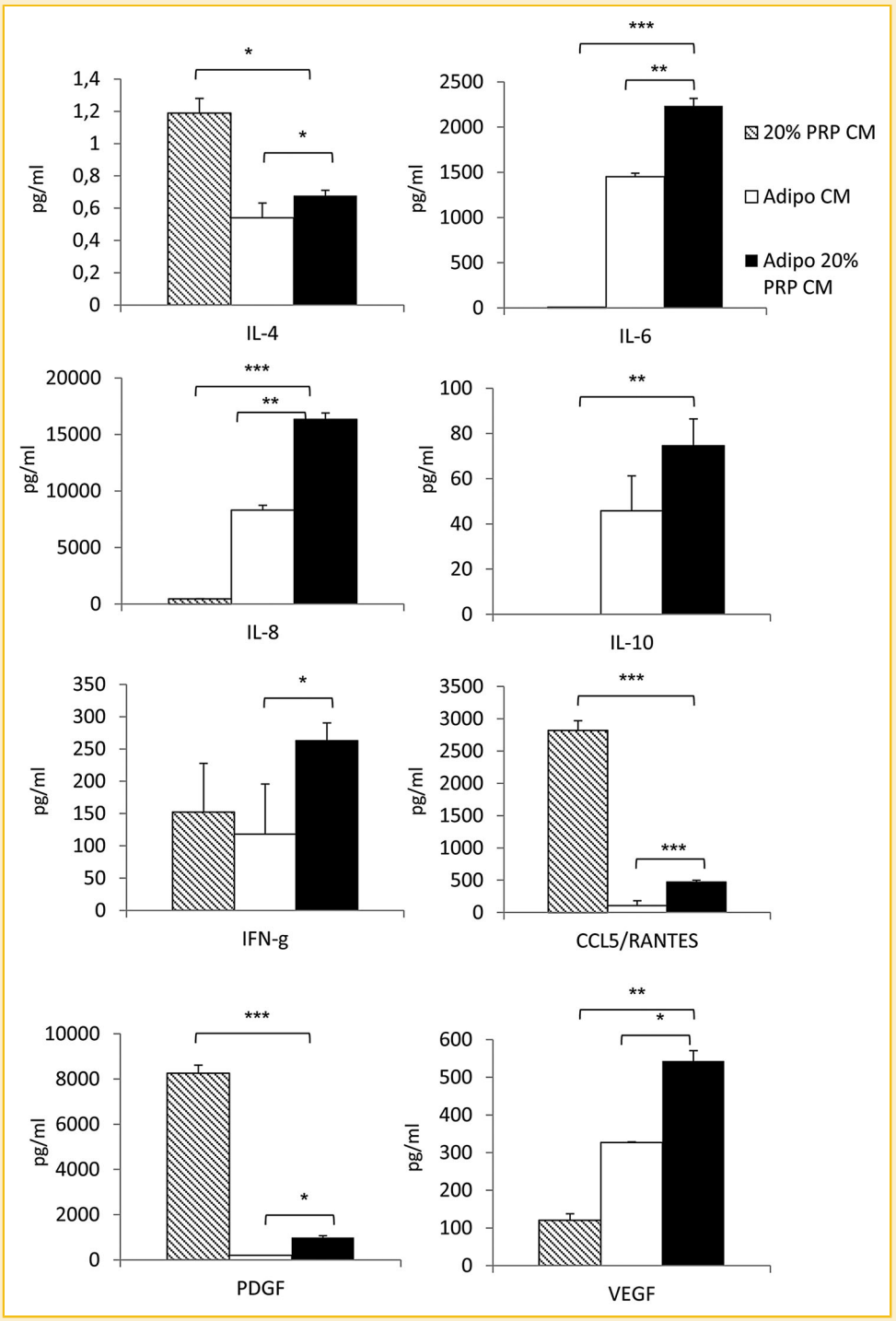

Fig. 4. Effect of PRP on adipocyte-released cytokines and growth factors. Human adipocytes have been incubated with PRP gel (20% vol/vol in DMEM F12 1:1) for 24 h. Media have been collected (CM) and tested by using the Bioplex multiplex Human Cytokine and Growth factor assay kit. Values ± SD are reported in the bar graph. Asterisks denote statistically significant values (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

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