

Effect of Platelet Lysate on the Functional and Molecular Characteristics of Mesenchymal Stem Cells Isolated from Adipose Tissue

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Abstract: *Background:* Mesenchymal stem cells (MSCs) are non-hematopoietic, adult, fibroblast-like, multipotent cells that are plastic adherent in standard culture conditions. They can be isolated from several tissues, but it is always necessary to expand them for clinical practice.

Aim: We investigated the effect of human platelet lysate (hPL) on the expansion of human MSCs isolated from adipose tissue (AT), comparing it with fetal bovine serum (FBS) and human platelet-poor plasma (hPPP).

Materials and Methods: Human AT-MSCs, hPL and hPPP were obtained from 7 healthy subjects. AT-MSCs were seeded at 1500 cells/cm² and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10% hPPP or 10% hPL. Cells were harvested, counted and analyzed by flow cytometry every 7 days for 5 passages (P). The differentiation assays, RNA isolation and co-culture with allogeneic lymphocytes were performed at the end of P2.

Results: AT-MSCs achieved a better proliferation rate when cultured with hPL than with hPPP or FBS (20 ±2 versus 8 ±3 and 6 ±3, respectively, at the end of P5 [p<0.01]). hPL preserved the differentiation capacity and typical expression of surface antigens, avoiding the risks associated with the use of animal derivatives. AT-MSCs demonstrated a stronger inhibitory effect on lymphocyte proliferation with hPL than with other culture conditions, even at a AT-MSCs:T cells ratio of 1:10. The transcriptional level of matrix metalloproteinase 2, used to evaluate stemness, was very high in all conditions tested.

Conclusions: hPL represents an effective and safe supplement for MSC expansion to be used in the clinical setting.

Keywords: Adipose tissue, culture medium, mesenchymal stem cells, platelet lysate, stem cells.

INTRODUCTION

Mesenchymal stem cells (MSCs) have the ability to undergo numerous cell divisions while maintaining an undifferentiated state (self-renewal) and the capacity to differentiate into specialized cell types (potency) [1-3]. Following the definition of a recent consensus conference [4], MSCs are adult, fibroblast-like, multipotent cells that are plastic adherent in standard culture conditions and generally express CD105, CD73 and CD90, lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules and differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*. Furthermore, MSCs have several immunoregulatory functions [5] that may contribute to the control of graft versus host disease (GvHD) after allogeneic hematopoietic stem cell transplantation [6]. Their interactions with hematopoietic stem cells (HSCs) influence their homing and differentiation, favoring bone marrow (BM) engraftment [7-9].

Bone marrow (BM) aspiration is still the major source of MSCs, although adipose tissue (AT) [10], trabecular bone [11, 12], Wharton's jelly of the umbilical cord [13], placenta [14] and umbilical cord blood [15, 16] have recently been reported as alternative, less invasive sources of MSCs. Adipose tissue represents an accessible, less invasive and rich source of MSCs [17]. MSCs obtained from AT (AT-MSCs) are known to express a range of cell surface antigens, but specific molecular markers that differentiate them from other MSCs have not yet been identified [18].

Because the number of AT-MSCs collected from tissues is insufficient to perform biological studies or to be employed in clinical practice, it is always necessary to expand the cells in culture media. Fetal bovine serum (FBS) is the most widely used culture medium supplement for *in vitro* studies and clinical applications. However, the use of FBS has recently raised concerns, as bovine proteins or peptides might be incorporated into MSCs during culture procedures [19], with the potential for viral or prion transmission and immune reactions. As a result, several countries have issued warnings and restrictions on the clinical use of cell therapy products prepared in the presence of FBS [20, 21]. Accordingly, the search for new, less immunogenic and safer media for culturing MSCs is warranted.

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Platelets are rich sources of multiple growth factors, most of which (PDGFs, TGF- β 1, bFGF, IGF-1) have been described to play critical roles in the regulation of wound healing. In fact, platelet concentrates have been used in clinical oral surgery applications to deliver growth factors in high concentrations to sites requiring bone grafting [22, 23]. We propose a culture system in which the growth factors required for AT-MSC proliferation and differentiation are provided by platelet-rich plasma.

In our study, we compared human platelet lysate (hPL), FBS and human platelet-poor plasma (hPPP) as culture medium supplements for AT-MSC proliferation and differentiation.

The effects of the three different media conditions on AT-MSC isolation, expansion and differentiation capacities were investigated according to their phenotypic and immunological characteristics. Gene expression profiles of AT-MSCs [24-26] were also explored.

MATERIALS AND METHODS

Cells Sample Collection, Supplements and Media

This study was approved by the local ethical committee.

Human MSCs were obtained from the adipose tissue of 7 healthy subjects (median age 40 years old, ranging from 25 to 50) undergoing abdominal plastic surgery. Samples were collected by surgical dissection of abdominal adipose tissue. AT samples and human plasma were obtained after donors signed informed consent forms, in accordance with the advice of the local ethical committee.

AT-MSCs were cultured under three different conditions. Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Sigma-Aldrich (S-A), St Louis, MO, US, <http://www.sigmaaldrich.com>) supplemented with 4 mM L-glutamine (S-A), 100 U/ml penicillin and 100 μ g/ml streptomycin (S-A) was the basal medium, which was supplemented with one of the following:

- 10% FBS (StemCell Technologies, Vancouver, Canada, <http://www.stemcell.com>) was used as the control medium;
- 10% human platelet lysate (hPL);
- 10% human platelet-poor plasma (hPPP).

Human plasma was obtained from heparinized blood of healthy AB blood donors. We decided to use pooled human AB serum from 5 blood donors to bypass inter-subject variability. To prepare hPL, samples were centrifuged at 240 g for 10 minutes; the median platelet count was 686×10^9 /L. Plasma were frozen and sonicated applying ultrasound to disrupt platelet membranes and release growth factors; remaining platelet bodies were eliminated by a high-speed centrifugation step for 30 minutes at 1500 g. Samples were mixed, aliquoted and stored at -20°C . To obtain hPPP [27], blood samples were centrifuged at 1500 g for 20 minutes. After centrifugation, hPPP was considered adequate when the platelet count was less than 3×10^9 /L. hPPP was sonicated for 2 minutes and re-centrifuged at 1500 g for 30 minutes to

eliminate remaining platelet bodies. Samples were mixed, aliquoted and stored at -20°C .

AT-MSC Isolation, Expansion and Colony Forming Units Assay

Samples were treated as reported by Zuk *et al.* [10]. Adipose tissue (approximately 100-200 ml) was washed with phosphate buffered saline (PBS; S-A) containing penicillin/streptomycin to eliminate blood contamination. It was then crumbled with sterile scissors and a lancet and digested with 0.1% collagenase A (Invitrogen, Carlsbad, CA, US, <http://www.invitrogen.com>) for 60 min at 37°C . The collagenase activity was neutralized by dilution with non-complete medium, and the stromal vascular fraction (SVF) was separated by centrifugation at 140 g for 10 min. The pellet was resuspended, filtered through a 70- μ m nylon cell strainer (Becton Dickinson Biosciences (BD), Franklin Lakes, NJ, US, <http://www.bd.com>) and centrifuged for 4 min at 270 g. Cells were resuspended in basal medium supplemented with (a) 10% FBS, (b) 10% hPPP or (c) 10% hPL and seeded at a density of 160×10^3 cells/cm² in cell culture flasks (BD). After 48 hours of incubation at 37°C in a humidified atmosphere containing 5% CO₂, cultures were washed with PBS to discard non-adherent cells and cultured until they reached 75-90% confluence, as observed using optical microscopy after approximately 8 days in culture; this phase is conventionally named the end of P0. At this time, cells were detached using 0.05% Trypsin/EDTA (S-A) for 5 minutes, harvested and reseeded at a density of 1500 cells/cm². Viability was determined by Trypan Blue (S-A) exclusion, and cells were counted using a Burkert chamber. After 3-4 days, non-adherent cells were removed, and the cultures were re-fed with fresh medium. Cells were then harvested repeatedly every 7 days until the end of passage 5 (P5) [28].

The frequency of colony-forming units (CFUs) AT-MSCs was determined by limit dilution assay of the SVF cell suspension. Cells were seeded in 6-well culture plates at concentration of 5×10^3 , 5×10^4 and 5×10^5 per well in the three different culture media. The plates were incubated at 37°C , 5% CO₂ for 15 days. At that time the plates were rinsed with PBS, fixed in formalin, stained for 20 minutes with Giemsa, rinsed with water and the colonies were counted (only when colonies contain >20 cells). These data were used to determine the number of CFUs AT-MSCs per million of SVF cells seeded.

Growth and Kinetic Determination

The population doubling (PD) was calculated for each passage (P) using the following equation: $\text{PD} = [\log_{10} N_1 - \log_{10} N_0] / \log_{10} 2$, where N_0 is the inoculum cell number and N_1 is the cell harvested number. The cumulative population doubling (cPD) was obtained by summing each population doubling per passage.

In Vitro Differentiation Studies

The capacity of AT-MSCs to differentiate into adipogenic and osteogenic lineages was evaluated at the end of P2 for all sample conditions. For the differentiation assays, cells were seeded at a density of 1×10^3 cells/cm² and cultured in

6-well culture plates using all three experimental conditions until they reached approximately 80% confluence. Then, the expansion medium was replaced using specific differentiation supplements.

To induce osteogenic differentiation, cells were incubated in medium containing 10^{-7} M dexamethasone, 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate (S-A). The medium was replaced twice per week. The formation of a mineralized matrix was confirmed by Von Kossa stain after 15 days: the cell layer was fixed with 5% formalin (S-A) and incubated with 1% silver nitrate solution (S-A) for 15 minutes in the dark. Later, 5% sodium thiosulfate was used to reveal hydroxyapatite deposits, and cells were counterstained for 2 minutes with neutral red. Differentiated cells displayed an accumulation of mineralized calcium phosphate [29] that appeared as dark deposits over the cell layer.

For the adipogenic lineage, the differentiation medium contained 10^{-6} M dexamethasone, 10 μ g/ml insulin and 100 μ g/ml isobutylmethylxanthine (S-A). The medium was replaced twice per week for 21 days. To visualize intracellular lipid vacuoles, cells were stained with Oil Red. Briefly, cells were fixed with 5% formalin for 5 minutes, colored with 0.5% Oil Red solution in isopropanol for 25 minutes and counterstained with hematoxylin for 2 minutes.

RNA Isolation and Real Time RT-PCR

AT-MSC stemness-related profiles and adipocyte and osteocyte differentiation were monitored with the expression levels of selected key genes. RNA isolation was performed at the end of the respective differentiation period. An undifferentiated cell sample was used as a negative control. Total RNA was isolated using the RNeasy[®] Mini Kit (Qiagen, Hilden, Germany). RNA contamination of genomic DNA was removed by treatment with the RNase-Free DNase Set (Qiagen). cDNA was synthesized using 500 ng of total RNA from each sample in 30- μ l reactions containing 300 U of M-MULV Reverse-Transcriptase (Promega, USA), 1x RT reaction buffer, 5 μ M random hexamer primers (Roche Diagnostics Corp., Indianapolis, USA), 1 mM each dNTP and 30 U RNase Inhibitor (Promega). One microliter of cDNA was then amplified by polymerase chain reaction (PCR; 7500 Real Time PCR System, Applied Biosystems, Foster City, CA) as follows: 40 cycles of a two-step PCR (95°C for 15 sec, 60°C for 60 sec) after uracil N-glycosylase (AmpErase[®], Roche) activation (50°C for 2 min) and initial denaturation (95°C for 10 min) with 1x Power SYBR[®] Green PCR Master Mix (Applied Biosystems). For transcriptional analysis, primer pairs mapping to the 3' untranslated region (3'-UTR) of human MMP2 (matrix metalloproteinase 2), PPARG2 (peroxisome proliferator-activated receptor gamma isoform 2), OCN (osteocalcin) and ALPL (alkaline phosphatase) mRNAs were chosen using Primer Express software v2.0 (Applied Biosystems). mRNA levels were normalized using the housekeeping gene ABL (Abelson murine leukemia virus). Primers were used at 300 nM final concentrations (primers sequence details are available upon request).

A simple panel of genes was chosen for real-time PCR (RT-PCR), including PPARG, ALPL, OCN and MMP2. PPARG mediates growth arrest at confluence during the dif-

ferentiation stage of adipogenesis [30] (isoforms PPARG1 and PPARG2, the latter restricted to adipose tissue). ALPL and OCN are osteoblast-specific marker genes, and OCN was described as a late marker of developing osteoblasts, appearing with matrix mineralization [31]. MMP2 play a major role in the directed crossing of human MSCs through the extracellular matrix barrier, which is present between cells of all tissue types [32, 33]. Recent evidence has demonstrated that constitutive expression of MMP2 essentially contributes to the ability of bone marrow-derived human MSCs to traverse human basement membranes [34].

Flow Cytometry

To analyze immunophenotypes, AT-MSCs were stained with various combinations of saturating amounts of monoclonal anti-human antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC). The following anti-human antibodies were used: CD105, CD44 and CD90 (Beckman Coulter, Fullerton, CA, USA, <http://beckmancoulter.com>) as mesenchymal markers, while CD45, CD31 and CD34 (Beckman Coulter) were used to exclude the presence of hematopoietic precursors or endothelial cells. Anti-human antibodies were combined as follows:

CD90-FITC/CD105-PE/CD45APC

CD44-FITC/CD105-PE/CD45APC

CD31-FITC/CD34-PE/CD45APC

Isotype control antibodies were used as negative controls.

ALDH

Aldehyde dehydrogenase (ALDH) enzymes are intracellular proteins that are selectively expressed in primitive hematopoietic cells. The fluorescent ALDH reaction product in viable cells measures enzymatic activity in these cells by comparing the fluorescence of a test sample with a control containing diethylaminobenzaldehyde, a specific inhibitor of ALDH.

At every passage, ALDH activity was tested with the ALDEFLUOR kit (Stemcell Technologies). To perform the assay, an aliquot of the substrate was added to 1×10^5 cells suspended in Aldefluor assay buffer. An aliquot of this cell mixture was transferred to a tube containing a specific ALDH inhibitor (negative control). All test and control tubes are incubated for 30-60 minutes at 37°C. The assay reaction was detected using a flow cytometer equipped with a 488-nm argon ion laser. The ALDH expression was measured on cells that were positive for CD105 and negative for the hematopoietic marker CD45.

Co-Culture of AT-MSCs with Stimulated Allogeneic Lymphocytes

To evaluate the inhibitory effect of expanded AT-MSCs on T cell proliferation *in vitro* and to show whether it was regulated by soluble factors or by contact-dependent mechanisms, we performed co-culture of AT-MSCs and allogeneic lymphocytes obtained with density gradient centrifugation. Lymphocytes/monocytes isolated by Ficoll gradient were seeded in a culture flask and, after monocytes adhesion of 3

hours, we verified whether the percentage of T cell was superior at 90%. To ensure efficient cell-to-cell contact, MSCs and lymphocytes were mixed and co-cultured in 96-well plates. For the soluble factor experiments, transwell chambers with a 0.4- μ m pore size PET track-etched membrane (Corning Costar, Cambridge, MA) were used to physically separate lymphocytes from MSCs, allowing the exchange of soluble factors only.

MSCs at the end of passage 2 (P2) were seeded in decreasing concentrations, 2×10^4 , 1×10^4 , 1×10^3 , 1×10^2 or 0 cells/well in 96-well plates and 1.2×10^5 , 6×10^4 , 6×10^3 , 6×10^2 or 0 cells/well in 24-well plates. After 24 hours, cell proliferation was blocked with 10 μ g/ml Mitomycin C, and cells were washed with complete medium. Human T cells from healthy donors were added to each well at a fixed density (10^5 /well in 96-well plates and 6×10^5 /well in the upper chamber of a transwell insert) to obtain the following MSCs:T cells ratio: 1:5, 1:10, 1:100, 1:1000 and 0:100000. These cells were stimulated with phytohemagglutinin (PHA). Co-cultures were incubated for 7 days, after which time T cell proliferation was measured with a BrdU proliferation assay, according to the manufacturer's protocol (Merck-Calbiochem, San Diego, CA, US, <http://www.merckbiosciences.com>).

Statistical Analysis

Data were presented as mean $\pm$ standard deviation (SD). A two-sided paired Student's t-test was used to analyze flow cytometry and cumulative population data to evaluate the

probability of significant differences. Differences were considered significant at $p < 0.05$.

RESULTS

Effects of hPPP and hPL on Cell Proliferation

In this study, we compared hPL, FBS and hPPP as culture media supplements for the expansion of AT-MSCs obtained from 7 healthy subjects. AT-MSC isolation from surgical specimens was successful for all subjects.

Cumulative population doublings were calculated for all passages to compare the effects of different supplements on cell proliferation. AT-MSCs showed a significantly higher cumulative PD when cultured with hPL (20 ± 2 at the end of P5) compared to hPPP and FBS (8 ± 3 and 6 ± 3 , respectively [$p < 0.01$]) (Fig. 1); we observed no significant difference between FBS and hPPP supplements.

With low density cultures performed in 6 well plates we established the number of clonogenic AT-MSCs included in the SVF in the presence of the three different supplement media. Our results confirm that hPL support the expansion of an higher number of individual colonies (median $1000/10^6$ SVF cells) compared to FBS (median $200/10^6$ SVF cells) but not significantly different from hPPP.

Differentiation Capacity

The differentiation of AT-MSCs (Fig. 2) into adipogenic cells was significantly influenced by the culture media supplements. The medium supplemented with FBS produced a

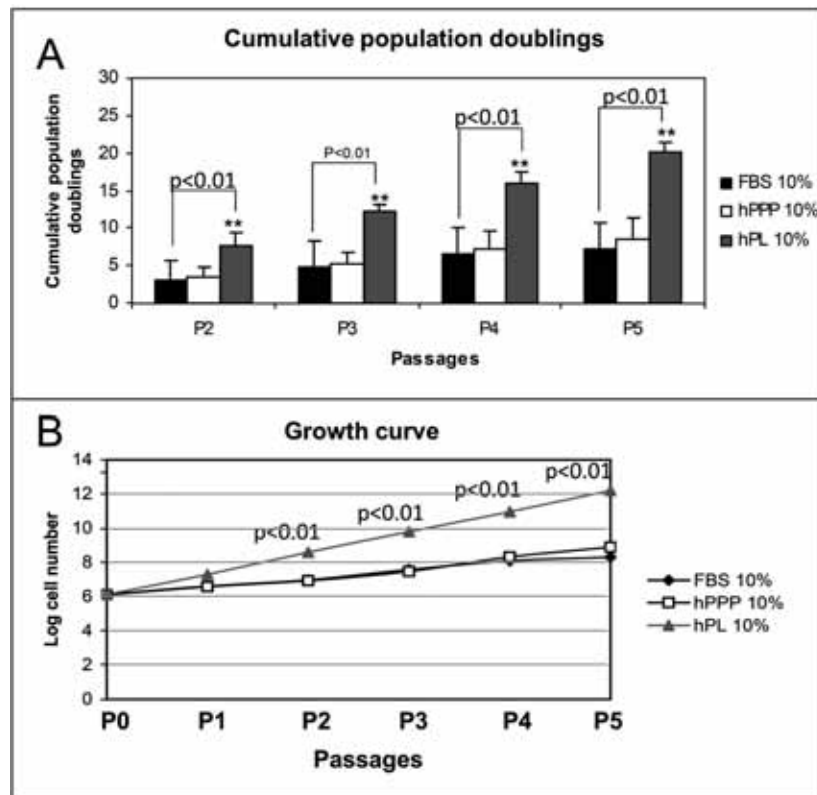


Fig. (1). (A): Mean cumulative population doublings for FBS (black), hPPP (white) and hPL (gray); $n=7$ for all three conditions until P5. (B): Growth curve indicating proliferation kinetics of AT-MSCs cultured in FBS, hPPP and hPL at every passages until P5. $p < 0.01$ in comparison to FBS.

much larger number of adipocytes than the medium supplemented with hPPP. By contrast, the medium supplemented with hPL resulted in rare or absent adipocytes.

The formation of a mineralized matrix, which reflects the differentiation potential of AT-cells into the osteogenic lineage, was strongly visible for the culture supplemented with hPL; it was absent for the culture supplemented with hPPP

and less evident for the culture supplemented with FBS (Fig. 2).

Flow Cytometry of AT-MSCs

All AT-MSC samples (n=7) were analyzed at every passage for the expression of surface proteins by flow cytometry (Table 1). More than 85% of AT-MSCs expressed CD105,

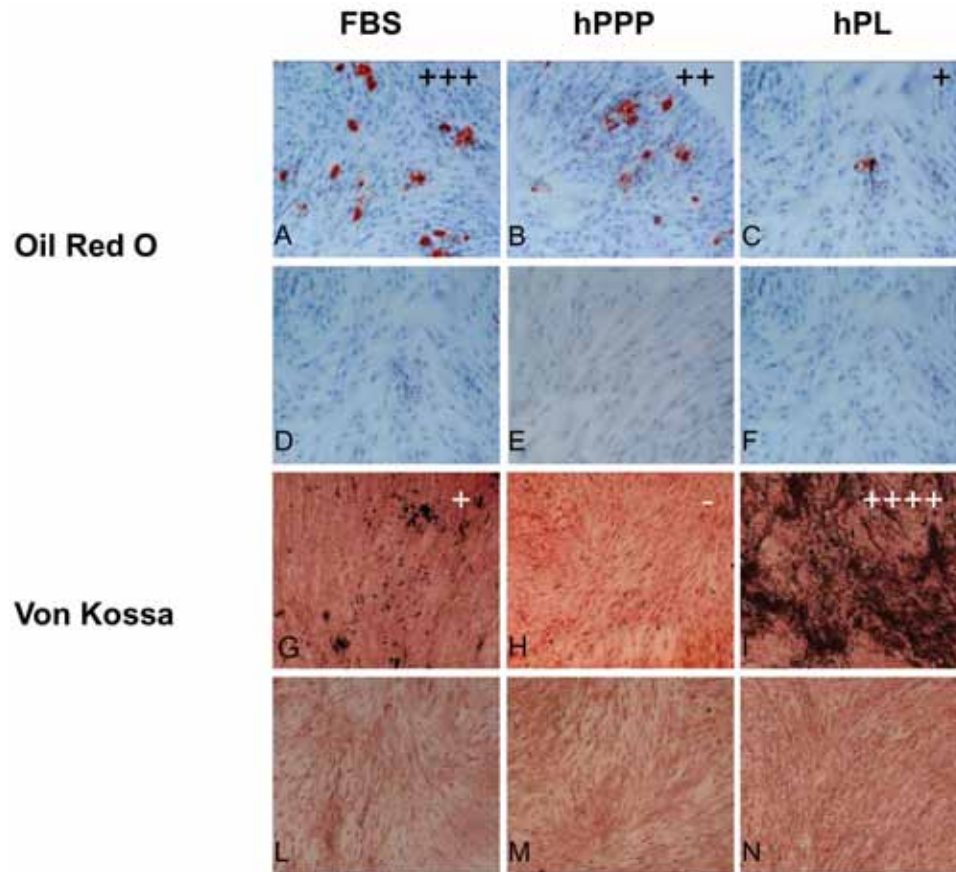


Fig. (2). Multilineage differentiation capacity of MSCs. Cells at P2 stage were investigated for their *in vitro* multilineage differentiation capacity. Adipogenesis was demonstrated by the formation of neutral lipid vacuoles colored by oil red O [A, B and C represent FBS, hPPP and hPL, respectively; D, E and F represent negative controls]. Osteogenic differentiation was demonstrated by the deposition of a mineralized matrix, visualized by the von Kossa stain [G, H and I represent FBS, hPPP and hPL, respectively; L, M and N represent negative controls]. All representative examples are shown at 100X magnification.

Table 1. Comparison of the Expression of Surface Proteins of MSCs Derived from AT Analyzed by Flow Cytometry

Antibodies	FBS (%) n=7		hPPP (%) n=7		hPL (%) n=7	
	P0	P5	P0	P5	P0	P5
CD45-/CD105+	86 ±8	nd	92 ±8	nd	92 ±7	nd
CD45-/CD105+/ALDH ⁺	18 ±10	12 ±6	30 ±18	21 ±17	23 ±9	19 ±7
CD45-/CD105+/CD90+	84 ±10	82 ±3	91 ±8	92 ±7	87 ±9	90 ±8
CD45-/CD105+/CD44+	85 ±11	80 ±4	90 ±10	95 ±5	91 ±9	88 ±4
CD45	3 ±1	0	3 ±1	0	2 ±1	0
CD31	1	0	1	0	2	0

Mean values of positive cells (%) ± standard deviation at the end of passage 0 (P0) and passage 5 (P5). ALDH⁺, CD90⁺ and CD44⁺ were established in a population of CD45⁺/CD105⁺ cells, while CD45⁺ and CD31⁺ cells were reported relative to the total number of cells analyzed. No significant differences were observed between FBS, hPPP and hPL supplements.

CD44 and CD90, and no significant difference in the expression of single markers was observed between FBS, hPPP and hPL supplements. These data confirmed the early isolation of a homogeneous population for all culture media conditions (Table 1).

We observed that ALDH activity remained constant in all passages (approximately 24% of cells were CD45⁺/CD105⁺), with no significant difference between the three culture conditions.

Direct Cellular Interaction is Essential for *In Vitro* Immunosuppressive Effects Mediated by AT-MSCs

When AT-MSCs and T-cells were co-cultured in close contact in the same well, the suppression of lymphocyte proliferation was proportional to the number of AT-MSCs. The inhibitory effect on lymphocyte proliferation was stronger ($p < 0.01$) for AT-MSCs when the ratio of MSCs:T cells was 1:5 (Table 2; Fig. 3).

By contrast, when MSCs and lymphocytes were separated by transwell membranes, we did not measure a relevant decrease in T cell proliferation at any MSCs:T cells ratio.

Molecular Studies and RT-PCR

The transcriptional level of MPP2, used as a candidate gene to evaluate stemness and known to be highly expressed in BM-derived MSCs, was compared to a human BM sample of a healthy donor cultured with FBS, hPPP and hPL. MMP2 expression in AT-MSCs was very high, even in samples that were not induced to differentiate, regardless of the experimental conditions. A maximal expression level (relative quantification, RQ = 14.3) was seen with FBS as a culture supplement, but high levels of MMP2 expression were also observed in samples cultured with hPL and hPPP (RQ = 8.7 and RQ = 6.7, respectively). As expected, PPARG2 was upregulated 12.8-fold in adipogenic differentiated AT-MSCs cultured in FBS. Transcript levels for ALPL (RQ = 3.6) and OCN (RQ = 2.6) of AT-MSCs supplemented with hPL are in

Table 2. Inhibition of PHA-Stimulated T Cell Proliferation by AT-MSCs in Media with Three Different Supplements in Cell Contact Co-Culture Test

Ratio MSCs:T cells	FBS 10% (RFU)	hPPP 10% (RFU)	hPL 10% (RFU)
0:100000 (negative control)	1591 ±276	2137 ±521	2350 ±459
1:1000	1424 ±185	1880 ±611	2216 ±329
1:100	1898 ±628	2381 ±697	1907 ±602
1:10	1015 ±274	1357 ±608	1514 ±535 ($p < 0.05$)
1:5	741 ±265 ($p < 0.01$)	787 ±294 ($p < 0.01$)	767 ±208 ($p < 0.01$)

Human MSCs from the adipose tissue of 7 patients were plated into 96-well plates (0, 100, 1000, 10,000 or 20,000 MSCs/well). After adhesion, MSCs were treated with mitomycin C, and 100,000 T cells from an unrelated donor were added in the presence of PHA (5 µg/ml). BrdU incorporation was measured 5 days after co-culture, and data were expressed as mean RFU (relative fluorescence unit) ±SD (all experiments were performed in duplicate). $p < 0.01$, $p < 0.05$ in comparison to 0:100.000 ratio (negative control).

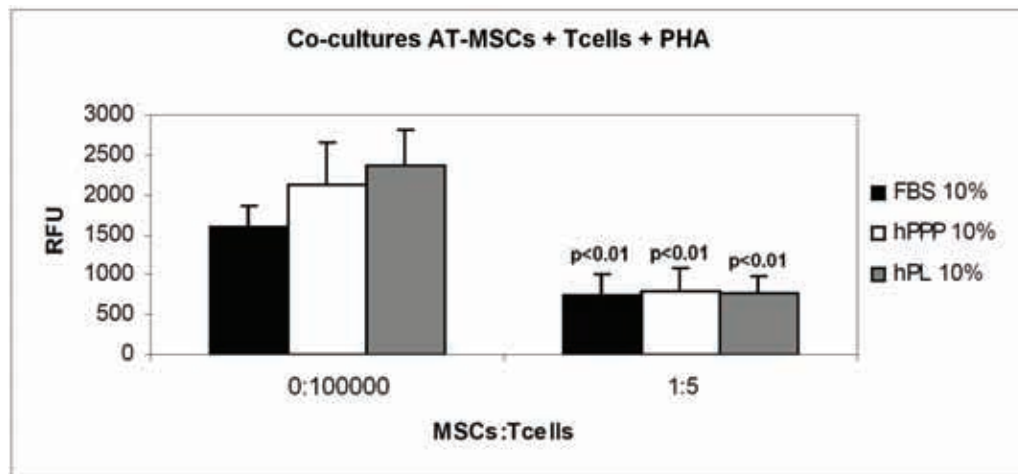


Fig. (3). The graph shows only significant results ($p < 0.01$) for cell contact co-cultures when the ratio of AT-MSCs:T cells is 1:5 (20,000 MSCs per well) in all culture conditions. $p < 0.01$ in comparison to 0:100.000 ratio (negative control).

accordance with morphological osteogenic differentiation results. The results of our RT-PCR analysis are summarized in Table 3. Beyond these data, in accordance with the differentiation assays, we noticed the abnormal and unexpected expression of the adipogenic marker PPARG2. PPARG2 was overexpressed in AT-MSCs expanded in hPPP and hPL media as well, although they were less morphologically differentiated. Table 4 shows that PPARG2 overexpression was present in all types of samples, even those cultured in osteogenic induction media. Similarly, the osteogenic marker ALPL was strongly upregulated in AT-MSCs supplemented with FBS (RQ = 31), despite a lack of morphological differentiation. A high level of inter-sample variability was observed for collected AT-MSC samples (Table 4).

DISCUSSION

With the present study, we showed that hPL can represent an effective and safe supplement in clinical settings because of the explosive and rapid growth obtained from hPL-supplemented cultures. Unlike FBS, which needs to be routinely pre-screened to guarantee both optimal MSC growth and the biosafety of the cellular product and can cause immune reactions in the host, hPL derived from monitored donors can be directly used in clinical settings.

In the cultures from our study, AT-MSCs had significantly higher cPD when supplemented with hPL (20 ± 2 ;

$p < 0.01$) compared to hPPP or FBS (8 ± 3 and 6 ± 3 , respectively; $p < 0.01$). In all three medium supplements, AT-MSCs grew with a spindle-shaped fibroblast-like morphology. However, the different supplements were able to modulate the multilineage differentiation capacity of MSCs. In fact, we observed that cultures supplemented with FBS produced a greater number of adipocytes compared to hPPP or hPL. The differentiation potential into the osteogenic lineage, however, appeared to be more pronounced with hPL compared to FBS or hPPP. This result could be due to the high concentration of growth factors in hPL, which may favor osteogenic conversion or a non specific deposition of calcium and reduce induction of adipogenic differentiation pathways.

Moreover, these different results could be explained by the fact that differentiation media have been developed to sustain the differentiation of BM derived cells. As a consequence, those media could be not optimized to differentiate AT derived cells.

Our molecular studies using RT-PCR were only partially in accordance with our morphological data, and some of the contrasting results may indicate that molecular data should be used with caution when monitoring MSC differentiation, as many variables (source, cytokines and media supplements) may influence differential gene expression. The strong upregulation of MMP2 by AT-MSCs suggests that

Table 3. RT-PCR Relative Quantification Values for AT-MSCs in FBS, hPL and hPPP-Supplemented Media

AT-MSC GENE EXPRESSION (N = 7)					
			FBS	hPL	hPPP
Gene	Reference	Sample	RQ (Min-Max)	RQ (Min-Max)	RQ (Min-Max)
MMP2	BM	NC	14.3 (7.9-25.8)	8.7 (6.5-10.0)	6.7 (5.5-8.9)
PPARG2	NC	AD	12.8 (11.4-13.7)	7.2 (2.3-19.5)	4.4 (2.1-7.4)
OCN	NC	OS	1.0 (0.9-1.1)	2.6 (1.2-4.2)	0.8 (0.7-0.9)
ALPL	NC	OS	31.7 (8.6-60.4)	3.6 (2.3-4.7)	2.6 (0.8-4.6)

Abbreviations: RQ, relative quantification; FBS, fetal bovine serum; hPL, human platelet lysate; hPPP, human platelet-poor plasma; NC, negative control (undifferentiated sample); AD, culture in adipogenic induction medium; OS, culture in osteogenic induction medium. MMP2, metalloproteinase-2; PPARG2, proliferator-activated receptor gamma-2 (adipogenic marker); GADPH, glyceraldehyde 3 phosphate dehydrogenase; OCN, osteocalcin (osteogenic marker); ALPL, alkaline phosphatase (osteogenic marker); BM, human bone marrow; NC, negative control (undifferentiated sample); AD, culture in adipogenic induction medium; OS, culture in osteogenic induction medium.

Table 4. Effect on PPARG2 of FBS, hPL and hPPP-Supplemented Media for AT-MSCs

PPARG2 GENE EXPRESSION			
		Supplements	
Sample	FBS	hPL	hPPP
	RQ (Min-Max)	RQ (Min-Max)	RQ (Min-Max)
AD	12.8 (11.4-13.7)	7.2 (2.3-19.5)	4.4 (2.1-7.4)
OS	7.0 (5.6-8.7)	8.3 (4.2-16.1)	5.2 (1.7-10.1)

Abbreviations: RQ, relative quantification; FBS, fetal bovine serum; hPL, human platelet lysate; hPPP, human platelet-poor plasma; AD, culture in adipogenic induction medium; OS, culture in osteogenic induction medium. Undifferentiated samples were used as a reference.

this gene might be stemness-related in this source, unlike what we observed in BM-MSCs. Thus, MMP2 gene expression could be used as a helpful tool to complete AT-MSC identification and expansion protocols. ALPL expression seemed to be enhanced by FBS, even if we did not morphologically observe a correspondent formation of a mineralized matrix. PPARG2 expression also did not agree with morphological data or with the largely known adipocyte-associated PPARG2 expression, suggesting a strong influence of media supplements in the induction of differential AT-MSC gene expression, beyond that found in source-related differences. A possible explanation might reside in the fact that samples grown in adipogenic and osteogenic induction media (Table 3) were influenced by the use of dexamethasone, which induced a non-specific effect on PPARG2 mRNA transcription. On the basis of these observations, we conclude that PPARG2 cannot be used as adipogenic molecular marker in *ex vivo* expanded AT-MSCs. In this regard, it will be necessary to reach new insights into the tightly regulated AT-MSC gene expression under various culture conditions.

MSCs marker proteins, such as CD44, CD90 and CD105, were highly expressed in all culture conditions, with no significant differences between FBS, hPPP and hPL. These results confirmed that marker expression is not influenced by these experimental conditions. Early on, we obtained a phenotypically homogeneous population of AT-MSCs in all culture media conditions, and no contamination of hematopoietic cells was observed after the end of the first passage.

It is well known that hematopoietic stem cells share elevated ALDH activity, which is proportional to the stemness status of the cell. Strategies based on measuring ALDH activity in combination with the expression of stem cell surface markers have been developed to characterize non-hematopoietic progenitors, as reported by Mitchell JB *et al.* [35]. A fluorescent substrate for this enzyme is used to identify, quantify and isolate hematopoietic cells with stem or progenitor characteristics [36, 37]. We evaluated ALDH activity of our AT-MSCs samples to determine if this method was reliable for identifying MSCs progenitors. The enzymatic activity of ALDH was inferior to that observed by Mitchell *et al.* [35], who showed that although ALDH levels were low in initial stromal vascular fraction cells, the percentage of ALDH enzyme activity reached >70% between passages P0 and P4. In our analysis, ALDH activity remained constant for all passages (approximately 24% of CD45⁻/CD105⁺ cells) and in the three different culture conditions, indicating that the three supplements did not alter enzymatic activity. Thus, we suggest that ALDH activity may not be fully representative for the definition of AT-MSCs.

Suppression of activated T-lymphocyte proliferation was dependent on the ratio of AT-MSCs:T-cells and was restricted to cell-to-cell contact conditions (96-well plates) for all three conditions. The inhibitory effect was more evident ($p < 0.01$) when the ratio of AT-MSCs:T-cells was 1:5. By contrast, when AT-MSCs and lymphocytes were separated by transwell membranes, we observed no relevant decrease in T-cell proliferation. Our results suggest that in all tested culture conditions, cell contact is fundamental for stem cell inhibitory effects. However, our observations differ from results obtained for BM-MSCs by other studies, which de-

scribed a suppression of T-cell proliferation both in cell-to-cell contact and transwell membrane conditions, although proliferation was lower in the latter condition [38-42]. The lack of T-cell inhibition in our transwell experiments could be due to the inhibition of some MSC activation pathways or to the inferior sensitivity of our detection method. Further studies will be necessary to clarify which mechanisms are involved in immunomodulation.

In our study, hPL increased the proliferation rate of MSCs in culture with respect to FBS and maintained the differentiation capacity and expression of the typical surface antigens, avoiding the risks linked to the use of animal derivatives. The variability of human plasma composition and platelets was easily bypassed using a pool of hPL from different donors. Finally, subcutaneous AT represented an alternative source of stem cells, with accessible, abundant and replenishable depots, providing a potential adult stem cell reservoir for each individual, even for patients undergoing sequential and prolonged chemotherapy treatments. For instance, from a SVF of 100 million of cells we obtained a number of AT-MSCs sufficient for clinical use, considering that to avoid aneuploidy cells shouldn't be expanded more than 3 passages. Since discordant effects have been obtained in the use of MSC in GvHD control in human, further studies will be necessary to clarify a possible *in vivo* translation.

ABBREVIATIONS

MSCs	= Mesenchymal stem cells
GvHD	= Graft versus host disease
BM	= Bone marrow
AT	= Adipose tissue
AT-MSCs	= MSCs obtained from AT
FBS	= Fetal bovine serum
hPL	= Human platelet lysate
hPPP	= Human platelet poor plasma
DMEM	= Dulbecco's modified Eagle's medium
PBS	= Phosphate buffered saline
P	= Passage
PD	= Population-doubling
cPD	= Cumulative population doubling
MMP2	= Matrix metalloproteinase
PPARG2	= Peroxisome proliferator-activated receptor gamma isoform 2
OCN	= Osteocalcin
ALPL	= Alkaline phosphatase
RT-PCR	= Real-time PCR
FITC	= Fluorescein isothiocyanate
PE	= Phycoerythrin

APC	=	Allophycocyanin
ALDH	=	Aldehyde dehydrogenase
PHA	=	Phytohemagglutinin
SD	=	Standard deviation
RQ	=	Relative quantification

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