Multipotent Mesenchymal Stem Cells with Immunosuppressive Activity Can Be Easily Isolated from Dental Pulp

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Background. Bone marrow mesenchymal stem cells (MSCs) are currently being investigated in preclinical and clinical settings because of their multipotent differentiative capacity or, alternatively, their immunosuppressive function. The aim of this study was to evaluate dental pulp (DP) as a potential source of MSCs instead of bone marrow (BM).
Methods. Flow cytometric analysis showed that DP-MSCs and BM-MSCs were equally SH2, SH3, SH4, CD29 and CD 166 positive. The in vitro proliferative kinetics of MSCs were measured by 3H-thymidine incorporation uptake. The immunosuppressive function of MSCs was then tested by coculturing PHA-stimulated allogeneic T cells with or without MSCs for 3 days.
Results. BM-MSCs could be differentiated in vitro into osteogenic, chondrogenic and adipogenic lineages. DP-MSCs showed osteogenic and adipocytic differentiation, but did not differentiate into chondrocytes. Although DP-MSCs grow rapidly in vitro between day 3 and day 8 of culture and then decrease their proliferation by day 15, BM-MSCs have a stable and continuous proliferation over the same period of time. The addition of DP-MSCs or BM-MSCs resulted in 91 ± 4% and 75 ± 3% inhibition of T cell response, respectively, assessed by a 3H-thymidine assay.
Conclusions. Dental pulp is an easily accessible and efficient source of MSCs, with different kinetics and differentiation potentialities from MSCs as isolated from the bone marrow. The rapid proliferative capacity together with the immunoregulatory characteristics of DP-MSCs may prompt future studies aimed at using these cells in the treatment or prevention of T-cell alloreactivity in hematopoietic or solid organ allogeneic transplantation.

Keywords: Mesenchymal stem cell, Dental pulp, Bone marrow, Immunomodulation.

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The role of bone marrow stroma in supporting hematopoiesis is well-recognized (*1*) and mesenchymal stem cells (MSC) (*2*) are an important component capable of differentiation into adipocytes (*3*), chondrocytes (*4*) and osteocytes (*5,6*) under appropriate experimental conditions. MSCs do not induce T-cell alloreactivity. In addition, MSCs display an immunoregulatory capacity by suppressing T-cell responses in *in vitro* and *in vivo* animal models (*7,8*). Due to these characteristics MSCs are good candidates for treatment of mesenchymal tissue disorders, and a potential use in the setting of autologous or allogeneic hematopoietic stem cell transplantation, gene therapy, organ transplant rejection and treatment of autoimmune disorders is envisaged. Recently the engraftment of allogeneic bone marrow-derived mesenchymal cells and stimulation of growth have been demonstrated in children with osteogenesis imperfecta (*9*).

MSCs have been obtained and expanded from placenta (*10*), bone marrow (*11*), human muscle (*12*), adipocytes (*13*) and other tissues, but more easily available alternative sources of cells expressing an ability to differentiate and to modulate the immune response are badly needed.

A promising source is adult dental pulp, a loose vascular connective tissue surrounded by dentine and consisting of a heterogeneous population of cells: the potential preodontoblasts, fibroblasts, stromal cells, endothelial and perivascular cells, neural cells and others.

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These cells maintain the homeostasis of dental mineralized tissues. Most adult pulp cells are postmitotic, but some cells may still divide and give rise to new pulp cells, able to differentiate even into odontoblasts and to form new dentine (*14*). All these cells and blood vessels are embedded in a specific rich extracellular matrix, which creates a microenvironment permitting repair processes (*15*).

In this work we have attempted to define the phenotype of DP-MSCs, and investigate their *in vitro* growth in comparison with bone marrow counterparts, after expanding them *ex vivo* and inducing them to differentiate *in vitro* into at least three types of cells: osteoblasts, chondroblasts and adipocytes.

Moreover our results demonstrated that multipotent MSCs from dental pulp display an increased immunosuppressive activity when compared to bone marrow mesenchymal cells.

MATERIALS AND METHODS

According to the policy approved by the local Ethical Committee, all tissue samples were obtained after informed consent.

Bone Marrow

Bone marrow was collected from five healthy adult volunteers (mean age 45 years; three males and two females) in heparinized tubes, diluted 1:3 with phosphate buffered saline (PBS) (BioWhittaker Cambrex, Walkersville, MD) and layered over a Ficoll-Histopaque gradient (1.077g/ml; Sigma, St. Louis, MO). The low-density mononuclear cells were washed twice in PBS, counted and plated at $1 \times 10^6/cm^2$ in culture flasks (Falcon BD, Bedford, MA, USA) in Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker Cambrex, Walkersville, MD) supplemented with 20% heat inactivated fetal bovine serum (FBS) (BioWhittaker Cambrex) and antibiotics (200 U/ml penicillin, 200 μg/ml streptomicin) (Sigma), and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 1 week, the nonadherent cells were removed by replacing the medium supplemented by 10% FBS. When the cultures were near confluence (after 2-3 weeks) the cells were detached by treatment with 0.05% trypsin-EDTA (Sigma) for cytometric analysis, functional assay, proliferation assay and immunological modulation study. MSCs were maintained and subcultured for up to 6-7 passages.

Dental Pulp

Vital human molars were obtained from five adult subjects (mean age 40 years; three males and two females), during routine dental extraction. After extraction, the tooth was immersed in physiological solution containing antibiotics to eliminate any contamination by the germs present in the oral cavity.

By means of pliers (bone forceps), the dental crown was fractured in several parts and the pulp uncovered. This procedure was performed soon after extraction, in sterile conditions, and required from eight to ten minutes to complete.

The tissue fragments were suspended in culture minimum essential medium (MEM) (Sigma) to which were added 25% inactivated FBS, antibiotics (200 U/ml penicillin, 200 μg/ml streptomicin) and 2.5 μg/ml amphotericin B (Sigma). This was then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for the time necessary to allow cells to slip down from the explants. In these experimental conditions the cells reached confluence within 15-20 days of culture.

The cell layer was removed by enzymatic digestion (0.1 % trypsin-EDTA) at 37°C and passaged on a surface three times greater.

To expand cells, MEM plus 10% FBS antibiotics was used. The medium was changed every 48 hr.

Flow Cytometric Analysis

For flow cytometric analysis, the fibroblast-like cells obtained from bone marrow and dental pulp were harvested at the same time of culture by treatment with 0.05% trypsin-EDTA and incubated with 1 μg/10^6cells FITC-conjugated antibodies for 40 min at 4°C in the dark. The antibodies used were: SH2, SH3 and SH4 (kindly provided by Dr. Mark Pittinger; Osiris Therapeutics, Baltimore, MD); anti-CD29 and anti-CD166 (Ancell, Bayport, MN); anti-CD14, anti-CD34 and antiCD45 (Becton Dickinson, San Jose, CA). After washing, cells were analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA) by collecting 10,000 events and the data analyzed using the Cell Quest Software (Becton Dickinson).

Functional Assays

At the third culture passage, DP-MSCs and BM-MSCs were induced to differentiate into three different types of

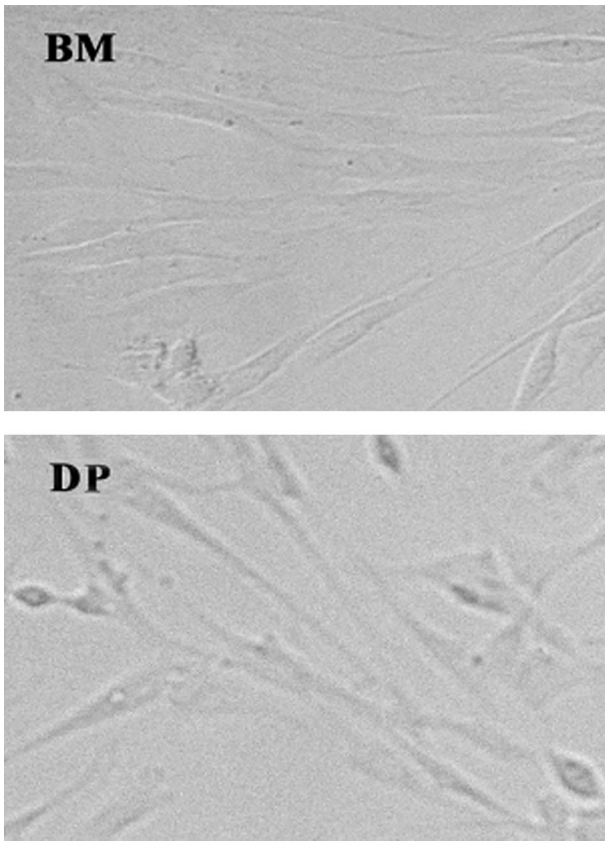


FIGURE 1. Photomicrographs showing mesenchymal stem cells primary culture of bone marrow (BM) and dental pulp (DP) after 3 weeks in culture.

cells: adipocytes, osteoblasts, chondroblasts, as previously reported (*2*).

Adipogenic Differentiation of MSCs

To induce adipocyte differentiation, 20×10^3 MSCs/cm^2 was cultured in DMEM high glucose (HG) (Sigma) supplemented with 10% FBS, 0.5 mM isobutyl-methylxantine (Sigma), 200 μM indomethacin (Sigma), 10^{-6} M dexamethasone (Sigma) and 10 μg/ml insulin (Sigma) in chamber slides (NUNC, Naperville, IL). The cells were cultured replacing the medium every 2-3 days. After 2-3 weeks of culture, the cells contained neutral lipids in fat vacuoles; they were fixed in 10% formalin and stained with fresh oil red-O solution (Sigma).

Osteogenic Differentiation of MSCs

To induce osteogenic differentiation, 3×10^3 cells/cm^2 were plated in chamber slides (NUNC) in α-MEM (Sigma) supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma), 0.2 mM ascorbic acid (Sigma), and 10^{-8} M dexamethasone (Sigma), and cultured for 3-4 weeks, replacing the medium every 2-3 days. To demonstrate osteogenic differentiation, the cultures were fixed and subjected to alkaline phosphatase and von Kossa staining.

Chondrogenic Differentiation of MSCs

To induce chondrogenic differentiation, aliquots of 2.5×10^5 cells were pelleted in polypropylene conical tubes in 0.5 ml of DMEM HG containing 6.25 μg/ml insulin (Sigma), 6.25 μg/ml transferrin (Sigma), 6.25 μg/ml selenous acid (Sigma), 5.33 μg/ml linolenic acid (Sigma), 1.25 mg/ml BSA (Sigma), 0.35 mM proline (Sigma), 1 mM sodium pyruvate (Sigma), 10^{-7}M dexamethasone (Sigma), 0.1 mM L-ascorbic acid-2-phosphate (Sigma), supplemented with 10 ng/ml transforming growth factor-beta 3 (TGF-β3) (R&D Systems, Minneapolis, MN). This medium was replaced every 3-4 days for 3-4 weeks. Pellets were formalin-fixed, embedded in paraffin, examined morphologically and immunostained for Type II collagen (Chemicon Int, Tamecula, CA). Type II collagen expression was also evaluated by RT-PCR.

Briefly, total cellular RNA was extracted from pellets (obtained as above described) and normal chondrocytes by TRIzol reagent method. First strand cDNA was synthesized with 100 ng of total RNA by Cloned AMV First-Strand cDNA Syntesis Kit (Invitrogen). The mixture was incubated for 1 hr at 50°C, heated for 5 min at 85°C. PCR amplifications were carried out in a thermal cycler with 2 μl cDNA from first-strand reaction, buffer 10×, $MgCl_2$ 1,5 mM, dNTP 0.2 mM, primers 0.2 μM and 1U of Taq DNA polymerase (Platinum *Taq* DNA Polymerase, Invitrogen). Primers were designed and synthesized for collagen type II (sense 5′-ACGGCGAGAAGGGAGAAGTTG -3′; antisense 5′-GGGGGTCCAGGGTTGCCATTG -3′). Each cycle included a denaturation step (94°C for 2 min), an annealing step (65°C for 30 sec), and an extension step (72°C for 1 min). The PCR products were visualized on a 2% agarose gel stained by ethidium bromide.

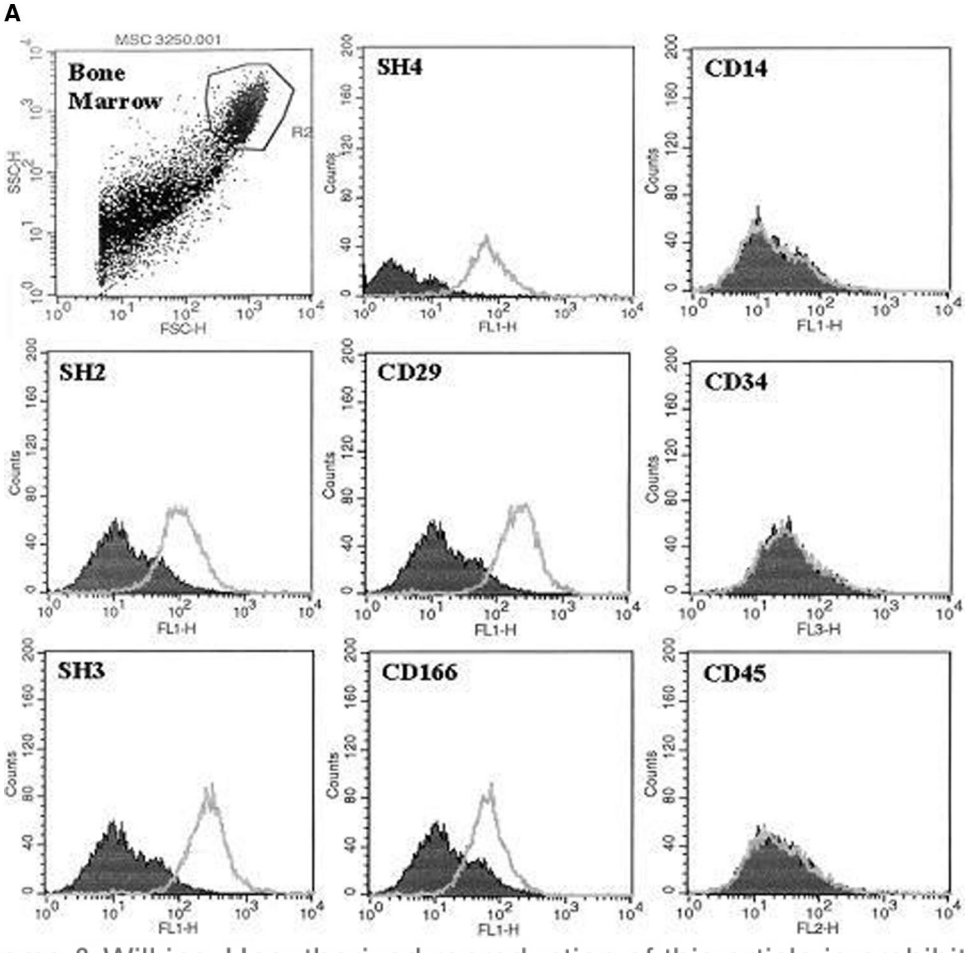
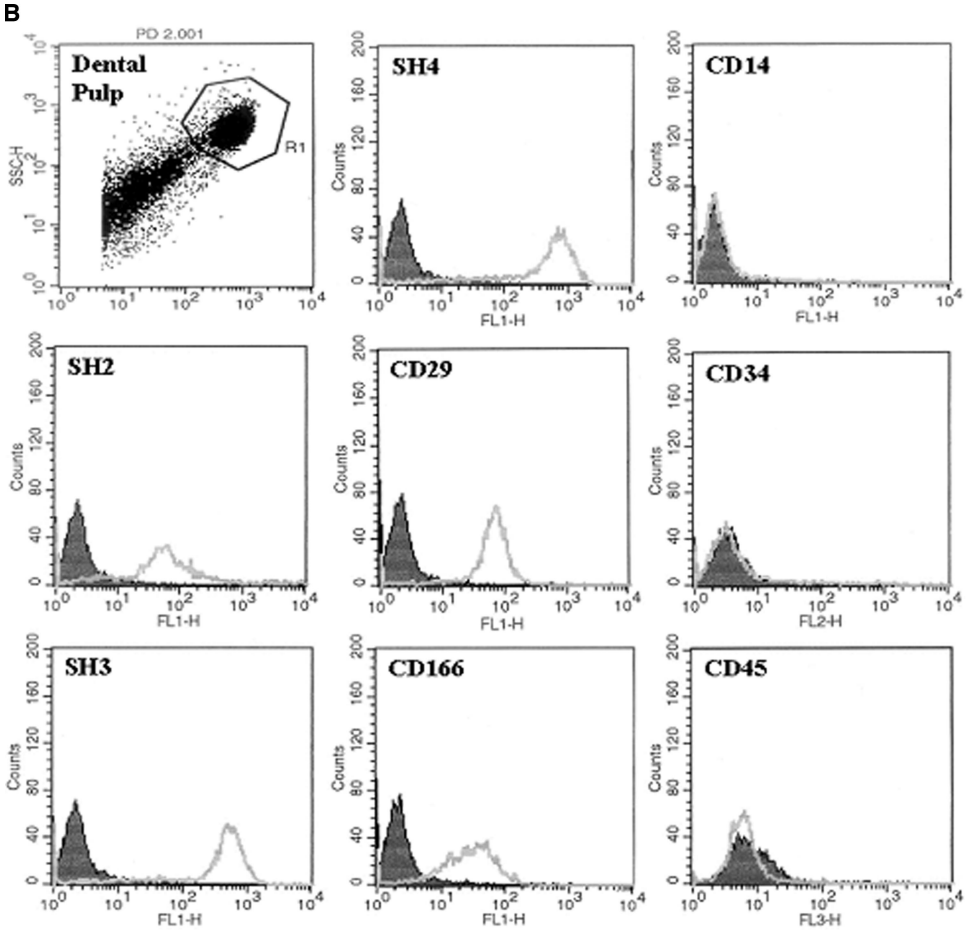


FIGURE 2. Flow cytometric plots showing the immunophenotype of bone marrow MSCs (A) and dental pulp MSCs (B). For analysis, cells were obtained from the homogeneous confluent monolayer at the second passage. The cells expressed SH2, SH3, SH4, CD29, CD166, while CD45, CD34, CD14 proved negative. Samples derived from three different subjects for each group were analyzed.



Proliferation Assay

To study the proliferation pattern of MSCs isolated from bone marrow and dental pulp samples, cells were treated with 0.05% trypsin-EDTA, washed in PBS and plated in DMEM 10% FBS at a concentration of 500/well in triplicate in a 96-well plate.

Before initiating the culture (day 0) and on days 1, 2, 3, 4, 8, and 15 of culture, MSCs were allowed to adhere overnight and then labeled with ^{3}H-Thymidine (^{3}H-TdR) (ICN Biomedicals, CA) at 0.037 MBq/well for 4 hr before cell harvesting using a multiple cell harvester (Skatron, Oslo, Norway). The cells were then assayed for incorporated radioactivity using a beta-counter (LKB, Uppsala, Sweden). The experiments were performed at least three times for each point described.

Immunological Modulation

Human T lymphocytes were isolated from peripheral blood of healthy donors by means of the MiniMACS high-gradient magnetic $CD2^{+}$(cell purity 96 $\pm$ 2%) cell sorting system (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions.

$CD2^{+}$ cells (5×10^{4}) were mixed with or without allogeneic irradiated (30 Gy) MSCs (5×10^{3}) in V-bottomed 96-well culture plates for 7 days in 0.2 ml modified RPMI-1640 medium (Sigma) containing 10% FBS. Phytohemagglutinin (PHA 2 mg/ml) (Sigma) was used to induce T-cell proliferation.

T-cell proliferation was measured on day 6 by means of a 12-hour pulse while ^{3}H-TdR (0.037 MBq/well) incorporation was measured by using a liquid scintillation counter. The experiments were performed at least three times for each point described.

Statistical Analysis

Differences in the proliferation patterns were analyzed by Student's *t* test.

RESULTS

MSC Isolation

Approximately 0.001% of BM cells harvested in the interface of the Ficoll gradient and allowed to adhere to the flask, showed a fibroblast-like morphology, and formed a layer after 2 weeks of culture.

A homogeneous population of adherent cells isolated from the DP-MSC culture is shown in Fig. 1. These cells are spindle-shaped and proliferate with a well-spread attached morphology. The same morphology appears in normal bone marrow cultures.

No difference in terms of sex or age was detected in

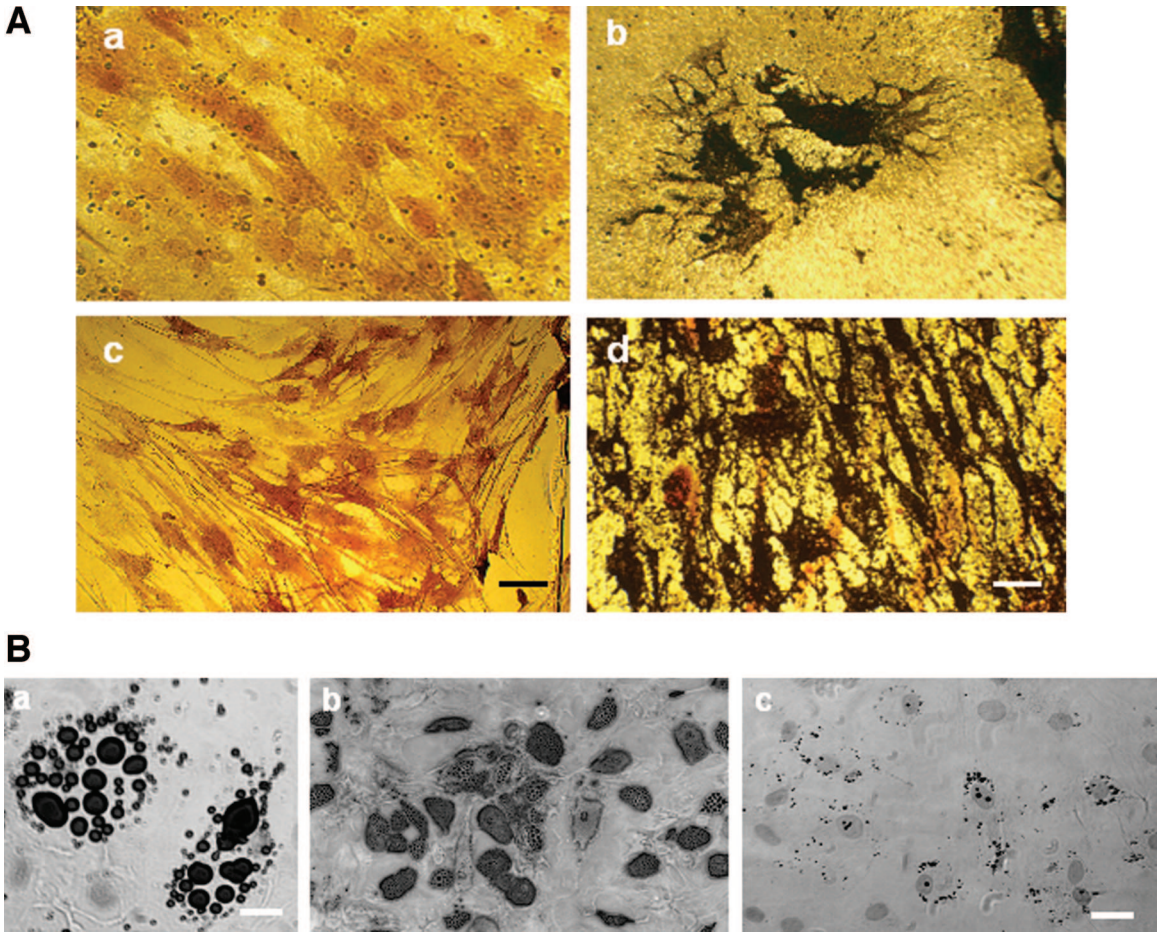


FIGURE 3. Ability of mesenchymal stem cells to differentiate to multiple lineages. (A) Osteogenic differentiation was indicated by the increase in alkaline phosphatase (a, c) and calcium deposition with Von Kossa staining (b, d). Bone marrow (a, b) and dental pulp (c, d); original magnification, 400×. (B) Adipogenic differentiation is visually marked by accumulation of neutral lipid vacuoles in cultures (red oil staining). Bone marrow adipocyte (a; original magnification 400×) bone marrow (b; original magnification 200×); dental pulp (c; original magnification 200×). Dental pulp mesenchymal stem cells showed a somewhat lower adipogenic differentiation.

isolating and characterizing the MSCs from the two different sources.

MSC Phenotype Characterization

The MSC phenotype was determined by flow cytometry utilizing antibodies against specific markers for mesenchymal stem cells kindly provided by Dr. Mark Pittinger (Osiris Therapeutics). In all, 96 ± 3% of cells selected from bone marrow samples expressed SH2, SH3, SH4 antigens which, where simultaneously present, were specific for MSCs (Fig. 2A), whereas markers specific for hemopoietic cell lineages (CD14, CD34, CD45) were negative.

The same antigen profile was expressed by 94 ± 4% of adherent cells deriving from dental pulp cultures (Fig. 2B).

MSC Differentiation

Mesenchymal stem cells isolated from bone marrow and dental pulp tissues were induced to differentiate into osteoblasts, chondroblasts and fat cells by using selective culture media as described in the materials and methods section. Bone marrow derived MSCs were capable of differentiating into all three lineages. In particular Von Kossa staining showing calcium mineral precipitation and the increase in alkaline phosphatase indicated osteogenic lineage (Fig. 3A), while neutral lipid was evidence of adipogenic differentiation (Fig. 3B).

DP-MSCs showed an osteogenic differentiation similar to BM-MSCs, a slightly less evident adipogenic differentiation, and a failure to differentiate into chondrocytes. To confirm this last result, RT-PCR was also performed and demonstrated the lack of collagen type II transcript (data not shown)

Proliferation Assay

MSC proliferation in cell culture conditions was measured by ^{3}H-thymidine incorporation. After overnight incubation, to let cells adhere to the flask, thymidine was added to the medium (time 0). As shown in Fig. 4, BM- and DP-MSCs showed strikingly different proliferation patterns over the time of observation. At each time, proliferation pattern of DP-MSCs was significantly higher than that of BM-MSCs. Moreover, although BM-MSCs showed a rather constant growth with a slight increase at day 15, DP-MSCs presented a

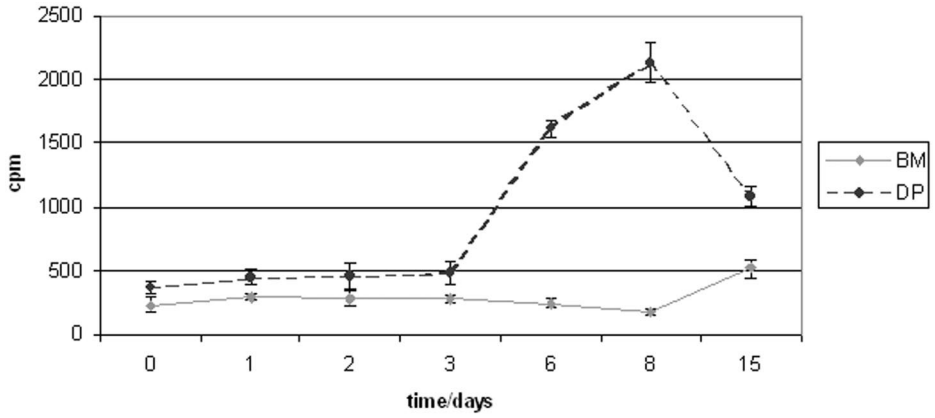


FIGURE 4. Comparison of proliferation assay for bone marrow and dental pulp derived mesenchymal stem cells. Cells were allowed to adhere overnight, then 3H-thymidine was added to the medium (time 0). Values represent the mean and SD of three separate experiments. At each time, differences in the proliferation pattern were statistically significant.

steep increase in radioactivity starting after day 3 ($P = 0.003$), continuing up to day 8 ($P = 0.0005$), and then declining, although on day 15 ($P = 0.005$) the radioactivity detected was still higher than at baseline.

Effect on T-cell Proliferation

Previous reports have demonstrated that BM-MSCs suppress T-cell responses (*16*). To evaluate whether DP also had a similar immunoregulatory effect on T-cell proliferation, purified CD2+ T cells were stimulated with PHA in the presence or absence of either DP- or BM-MSCs. In Fig. 5 it is demonstrated that both MSC cell populations prevented T-cell response to PHA, resulting, on average, in a 75 ± 3% and 91 ± 4% inhibition, respectively (data are derived from three experiments).

DISCUSSION

Adult bone marrow hemopoietic stem cells possess well-known multipotent capacities. The hypothesis that certain adult stem cells might have greater potential first came from the observation that in human bone transplants, donor cells were subsequently found in various different recipient tissues (*17*). Normal hematopoiesis depends on the close cooperation between hemopoietic progenitors and MSCs (usually present in the bone marrow stroma) for optimal proliferation and differentiation. Experimental and clinical data indicate that MSCs have a large potential for differentiating into various tissues in bone marrow- and organ transplantation and in gene therapy, owing to their plasticity under appropriate conditions and their immunomodulatory properties. It would be very important if it were found that mesenchymal stem cells deriving from various body tissues approach the plasticity of embryonic cells (*18–23*).

In this respect the recognition of easily accessible new sources and the improvement of methods for ex vivo isolation, expansion and differentiation of mesenchymal stem cells, whatever the source, have become crucial steps.

We looked into the potential of adult dental pulp as a source of MSCs because it is a vascular connective tissue similar to mesenchymal tissue. In plating DP-MSCs in culture, we obtained a layer of fibroblast-like cells from which we were able to isolate a cell population whose phenotype was similar to that of bone marrow derived MSCs. In fact, the cells expressed mesenchymal progenitor-related antigens SH2, SH3, SH4, CD166 and CD29 with a cellular homogeneity of 90-95%. Expression of endothelial antigen was not being sought in our experiments, but it was reported that BM-MSCs and DP-MSCs resided in the perivascular niches of bone marrow

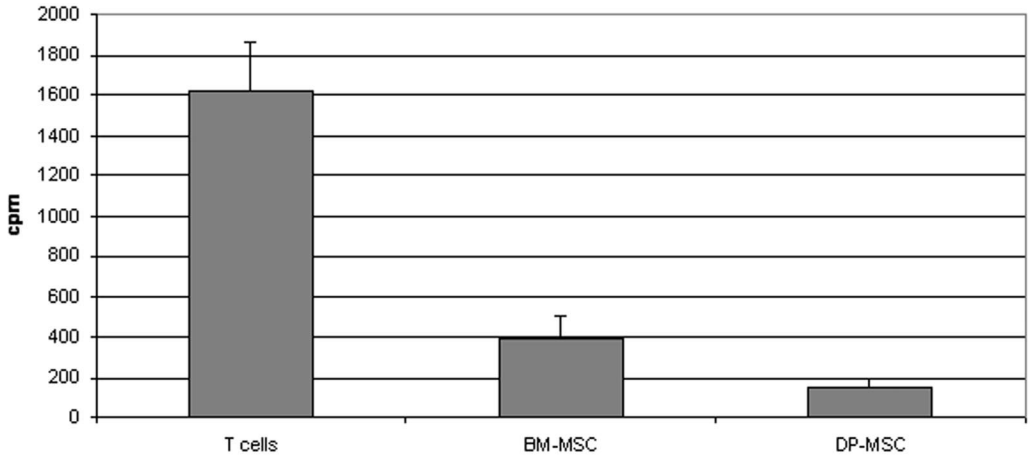


FIGURE 5. Inhibitory effect of mesenchymal stem cells on T-lymphocyte proliferation. The lymphocyte activity was reduced to 25% and 9% in the presence of bone marrow and dental pulp mesenchymal stem cells, respectively. The experiments were performed at least three times for each point described.

and dental pulp (*24,14*) and expressed pericyte- and pericyte-associated antigens, but not von Willebrand factor (*24*), which rules out any endothelial origin for the cells. Moreover, the cell populations had a similar gene expression profile (*25*).

When compared to BM-MSCs, DP-MSCs presented a higher proliferation pattern and lower differentiation ability. The most evident difference was the inability of DP-MSCs to differentiate towards chondrogenesis, as shown by fluorescent immunostaining and RT-PCR. This may indicate either that BM-and DP-MSCs are present at different stages of commitment and differentiation, not marked by phenotypical characteristics, or that different humoral networks are involved in each microenvironment.

On the other hand, our results indicated that DP-MSCs inhibit the proliferation of PHA stimulated T-cells, the effect being stronger than in BM-MSC and T-lymphocyte co-cultures previously described for their immunomodulatory effects (*16,21,26,27*).

Recently Le Blanc et al. (*28*) demonstrated that a grade IV acute graft versus host disease was controlled by the infusion of third-party haploidentical bone marrow mesenchymal cells, as these are not immunogenic and possess a specific capacity for inhibiting T-cell proliferation. In this respect, the rapid proliferative kinetics of DP-MSCs, as compared to their bone marrow counterparts, might represent an immunosuppressive advantage when treating immune reactions, such as graft rejection or graft-versus-host disease, in view of their potentially greater effect.

In conclusion, DP-MSCs are derived from a very accessible tissue resource, which is further expandable by using deciduous teeth, and possess stem cell-like qualities, including very good self-renewal and multilineage differentiation. Their capacity to induce osteogenesis (*29*) could obviously be utilized in paradontology and implantology. Moreover DP-MSCs could have potential clinical application in autologous *in vivo* stem cell transplantation for calcified tissue reconstruction. Their proven immunomodulatory activity makes DP-MSCs suitable for suppression of T-cell mediated reaction in the setting of allogeneic bone marrow transplantation.

We plan to bank mesenchymal stem cells from various different sources, including DP-MSCs, for future experimental and clinical use, in light of the results of the present work.

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