Human dental pulp stem cells conditioned medium for bone regeneration: A preliminary in vitro study.

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Abstract

Regenerative medicine and tissue engineering use a combination of scaffold, stem cells, and bioactive molecules to repair and restore the function of damaged tissues or organs. Secreted factors, alone without the stem cells themselves, may be sufficient to achieve regeneration. Such secreted factors can be collected in the medium where stem cells are cultured; this recovered supernatant is called conditioned medium. Stem cell-derived conditioned medium has a promising prospect to be produced as pharmaceuticals for regenerative medicine, as it avoids the legislative barriers linked to clinical stem cell use.

This preliminary study was conducted to test the in vitro influence of human dental pulp stem cells conditioned medium (hDPSC-CM) on osteoblast proliferation, maturation, and mineralization. MG-63 osteoblast cells were studied in presence and absence of 50% hDPSC-CM over a period of 3 weeks. Cell proliferation was assessed by cell counts during the first three days of culture. Specific bone cell markers: alkaline phosphatase (ALP), type I collagen (Col), and osteocalcin (OC), were studied by immunostaining and mineralization was evaluated by histological staining (alizarin red).

During the initial culture steps, hDPSC-CM significantly increased osteoblast proliferation. hDPSC-CM also enhanced osteoblast maturation and further mineralization. Immunostaining of bone mineralization markers (ALP, OC, and Col) tended to show an increased maturation of osteoblast in presence of hDPSC-CM. Calcium deposits stained by alizarin red after 3 weeks were also more abundant when osteoblasts were cultured in hDPSC-CM.

This study provides encouraging preliminary data about positive influence of hDPSC-CM on osteoblast proliferation, maturation, and mineralization, confirming its potential role for bone regeneration.

1. INTRODUCTION

Bone tissue engineering after maxillofacial trauma aims to provide new therapeutic solutions in the field of regenerative medicine to significantly improve facial reconstruction. Tissue engineering consists of associating a matrix with mesenchymal stem cells and bioactive molecules. Nevertheless, different difficulties for this combined medical device (advanced therapy medicinal product) make it difficult to predict its possible marketing[1]. Studies have shown that the beneficial effects of different stem cell-based cellular therapy is actually due to the ability of these cells to secrete trophic factors that exert a positive impact on damaged tissues, rather than to their differentiation ability to transform into necessary cells[2-3]. Various studies on cell-derived secreted factors have shown that these secreted factors alone are sufficient to cause tissue repair, even without the use of stem cells[4]. These factors are found in the medium where stem cells are cultured. After centrifugation, the obtained medium, containing the secreted factors only, is called the conditioned medium (CM)[5]. The use of CM has

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several advantages over the use of stem cells; it can be manufactured, lyophilized, packaged and transported easily. Furthermore, since it is cell-free, there is no need to match the donor and the recipient to avoid rejection problems[6]. In addition, its use could be a viable alternative to stem cell transplantation, which is often hampered by low efficacy and its carcinogenic potential after grafting. For all these reasons, many teams are currently focusing on the use of CM.

In this study, our aim was to evaluate the influence of conditioned medium derived from human dental pulp stem cells (hDPSC-CM) on proliferation, maturation, and further mineralization of human MG-63 osteoblast-like osteosarcoma cells.

Osteosarcomas are malignant bone tumors and osteosarcoma-derived cells are commonly used for osteoblastic models. "MG-63 osteoblast-like osteosarcoma cells" is one of the osteossarcoma cell lines used in research.

2. MATERIALS AND METHODS

2.1 Isolation and culture of dental pulp cells -DPSCs

Human impacted third molars extracted for orthodontic reasons were recovered from healthy patients (15−18 years of age). Written informed consents were obtained from patients' parents. The present protocol was approved by the local ethical committee (Comité de Protection des Personnes, Montpellier Hospital, France). Dental pulp stem cells were recovered as previously described[7]. Briefly, teeth were disinfected with chlorhexidine and dissected with a piezotome at the cementoenamel junction. Under sterile conditions, pulp tissues were gently separated from crown and root. Pulps were submitted to enzymatic digestion in a collagenase-dispase solution (3 mg/ml collagenase and 4 mg/ml dispase) that hydrolyzed the tissue structure proteins and allowed the collection of pulp cells only. After one hour of incubation, the solution was immersed in αMEM* supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and filtered through 70 µm Falcon cell strainers. Culture medium was changed after 24 hours to remove non-adherent cells and later twice a week. Cells were passed when they reached 90% confluence.

** αMEM = Minimum Essential Medium Eagle-alpha modification (Alpha MEM) with nucleosides.*

2.2 DPSC Characterization

Subconfluent cells were collected and analyzed for minimal criteria to define human mesenchymal stem cells, such as adherence to plastic, expression of cell surface antigens, and the ability to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro[8]. Antigen profiles of cultured DPSCs were analyzed by detecting the expression of the cell surface markers CD90, CD146, CD117, and CD45 (Fig. 1C) using flow cytometry (all antibodies were provided by Miltenyi Biotec, Paris, France).

2.3 Preparation of hDPSC-CM

At the fourth passage, cells were divided in 75 cm² flasks with conventional culture medium. At 70% confluence (Fig. 1A), the medium was removed from each flask and cells washed with phosphate-buffered saline (PBS) twice, then 10 ml of fresh (serum-free) medium were added. After 48 hours, the medium was collected having DPSC-secreted factors (the conditioned medium); it was later submitted to centrifugation at 1500 rpm for 5 minutes, then at 3000 rpm for 3 minutes. Supernatant was collected after each centrifugation. The conditioned media were used directly.

2.4 Culture of osteoblasts

MG-63 osteoblast-like osteosarcoma cells were obtained from the European collection of cell cultures. Frozen ampoules were transferred to the 37 °C water bath for 1 to 2 minutes. The contents were transported by pipette into a 75 cm² flask containing 12 ml of the culture medium. The conventional culture medium of MG-63 is composed of Eagle's minimal essential medium (Sigma-Aldrich), 1% glutamine, 1% non-essential amino acids (Sigma-Aldrich), 1% penicillin-streptomycin (PS) and 10 % fetal bovine serum (FBS). After 2 days, MG-63 were confluent, they were then passed and used (Fig. 1B).

2.5 Proliferation of MG-63 cells

In order to test the proliferation of MG-63 cells, they were transferred into plates at a density of 50,000 cells/well, in the presence of: (1) 50% of conventional MG-63 culture medium + 50% hDPSC-CM, (2) 50% conventional MG-63 culture medium with 10% FBS

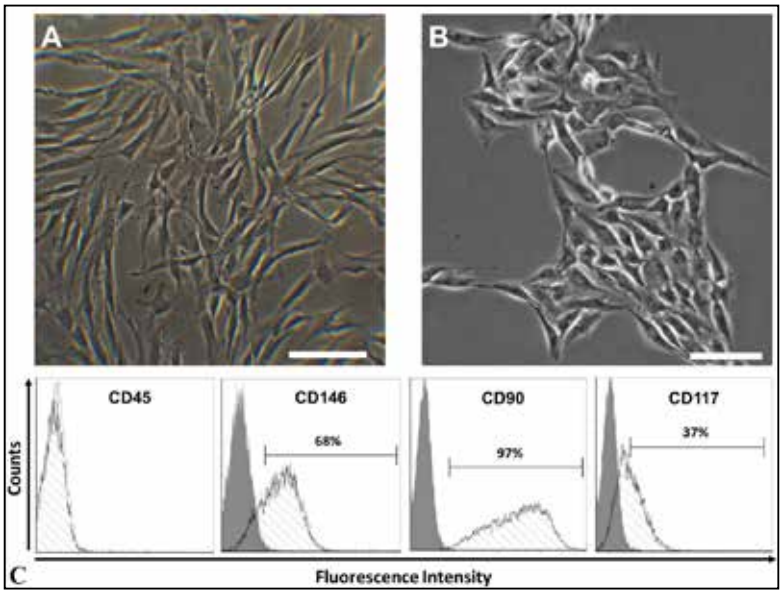

Fig. 1. Images of (A) Human dental pulp stem cells, reaching 70% of confluence and (B) MG-63 in culture, observed under phase contrast microscopy with 32× and 20× magnification respectively. Scale bar: 100 µm. (C) Flow cytometry analysis of subconfluent dental pulp cells. Single-parameter histograms showing the expression of markers CD 45, CD 146, CD 90, and CD 117.

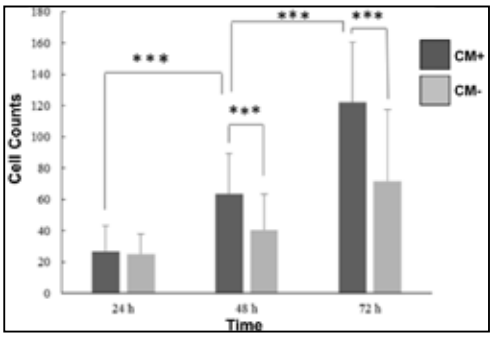

Fig. 2. Cell counts after 24 hours, 48 hours, and 72 hours of incubation with or without 50% hDPSC-CM. Cells counted on areas measuring 2362×1325 µm. Experiment made in triplicate, with 13 measurements per sample.
****p <0.001.*

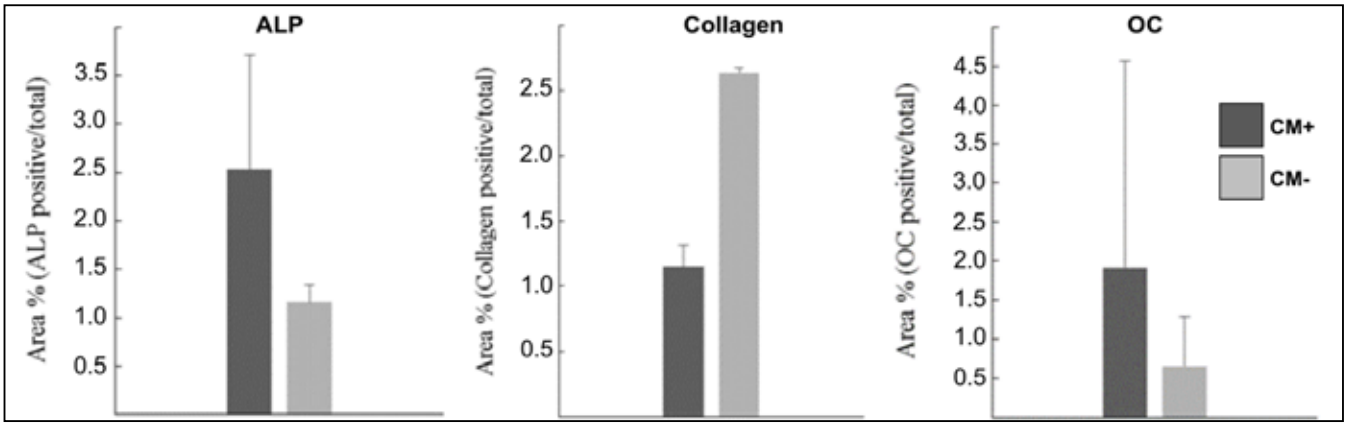

Fig. 3. The effect of DPSC-CM on the formation of ALP positive cells after 10 days, the mineralization of col and the formation of OC after 21 days in MG-63 cultures. Image analysis was performed by measuring the area covered by ALP, Col and OC positive cells from 2 replicate cover slips for each one. When compared to the control, the p values were 0.333, 0.333 and 1 with 50% DPSC-CM for ALP, Col, and OC respectively.

+ 50% alpha-MEM without serum (to have the same percentage of FBS in both samples). Cells were counted after 24 hours, 48 hours, and 72 hours of incubation: cells were fixed with 4% paraformaldehyde and stained with DAPI** (to stain nuclei). Experiments were made in triplicate, with 13 measurements per sample in attempt to cover the surface. Images were taken by an epi-fluorescence microscope, at 10x magnification and analyzed with ImageJ software (National Institutes of Health -NIH, Bethesda, Maryland, USA). Results were presented as mean ± standard deviation, with the error bar representing standard deviation.

2.6 Alkaline phosphatase (ALP), type I collagen (Col), and osteocalcin (OC) staining

For ALP evaluation, 200,000 cells/well were cultured in a 6-well plate under the same conditions (1) and (2) monitored for multiplication. At day 9, immunohistochemical analysis was performed. For OC and collagen, osteoblasts were transferred into glass petri dish at a density of 200,000 cells / petri dish in the presence of: (3) 50% classical MG-63 culture medium +50% hDPSC-CM +5Mm β-glycerophosphate calcium, (4) 50% conventional MG-63 culture medium with only 5% FBS + 50% DPSC conventional culture medium with only 5% FBS + 5Mm β-glycerophosphate calcium. A 50% proportion of hDPSC-CM was chosen similarly, as for the proliferation tests. β-glycerophosphate calcium was added to help MG-63 mineralization induction. Immunocytochemistry was tested after 21 days. Experiments were conducted in duplicate.

Osteoblasts MG-63 were fixed with paraformaldehyde 4%, rinsed with PBS, and covered with blocking buffer solution (BSA 1% + 0.3% Triton X-100) at room temperature overnight. Later on, they were incubated for one hour at room temperature with conjugated antibodies (for ALP, collagen, OC) diluted 1:100 in a blocking buffer solution. Images were taken by an epi-fluorescence microscope, at 10x magnification and analyzed with ImageJ by measuring the area covered by ALP, Col, and OC positive cells. Results were presented as mean ± standard deviation, with error bar representing standard deviation.

*** DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent stain that strongly binds to A-T rich regions in DNA. This stain is extensively used in fluorescence microscopy.*

2.7 Alizarin red staining

A total of 100,000 cells/well were seeded in 24 well plates under the same conditions as for OC and collagen. After 21 days, cells were rinsed with PBS and fixed with 95% ethanol for 30 minutes at room temperature. Then, they were washed with water twice and incubated with a 2% alizarin red solution at a pH between 4.1 and 4.3 for 5 minutes. They were then rinsed 5 times with distilled water and observed rapidly. Experiments were conducted in triplicate.

2.8 Statistical analysis

Statistical analyses were performed using SigmaStat software (SigmaStat 4.0, Systat Software Inc., Chicago, Illinois, USA). Normality was tested by Shapiro-Wilk test. Then, depending on it, we performed either Student t-test or non-parametric Mann-Whitney test.

The data of cell proliferation at 24 and 48 hours were compared with Student t-test. Those at 72 hours were compared with Mann-Whitney test, as the normality test was failed. The Mann-Whitney test was used also to compare the data of ALP activity, collagen, and OC expression.

Quantitative data were expressed as mean ± SD. A p-value less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1 hDPSC-CM stimulates osteoblast MG-63 proliferation

Any significant difference was observed, at 24 hours, among samples with or without hDPSC-CM. After that, the hDPSC-CM increased significantly (***$p<0.001$) the number of MG-63 cells compared to the control (63.7 ± 25.4 vs. 40.3 ± 22.8 cells) at 48 hours and (122.1 ± 38.2 vs. 71.6 ± 46) at 72 hours (Fig. 2).

3.2 hDPSC-CM enhances insignificantly MG-63 ALP, Col, and OC expressions

The ALP and OC amount increased with hDPSC-CM (0.02 ± 0.01 vs. 0.01 ± 0.001 control and 0.0019 ± 0.02 with hDPSC-CM vs. 0.006 ± 0.006 control respectively), and the non-mineralized collagen decreased (0.002 ± 0.0003 with hDPSC-CM vs. 0.005 ± 6.95E-05 control), but both evolutions were not significant compared to the control (Fig. 3).

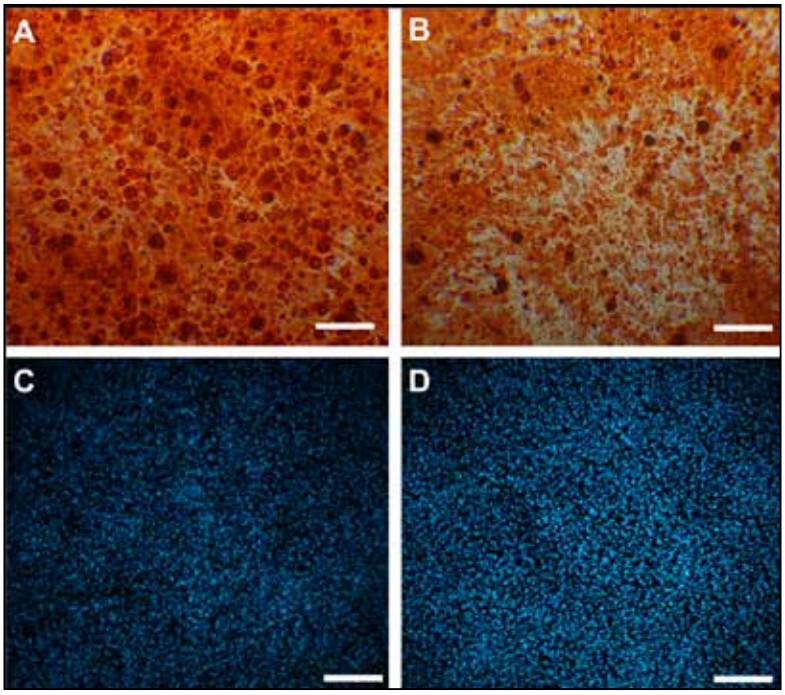

Fig.4. Calcium nodules (in brown) formed after 21 days in the presence (A) or no (B) of 50% hDPSC-CM, stained with red alizarin and observed under light microscope with 10× magnification. Corresponding DAPI staining images of cells growing with (C) or without (D) hDPSC-CM show the same compact MG-63 layer. Scale bar=200 µm.

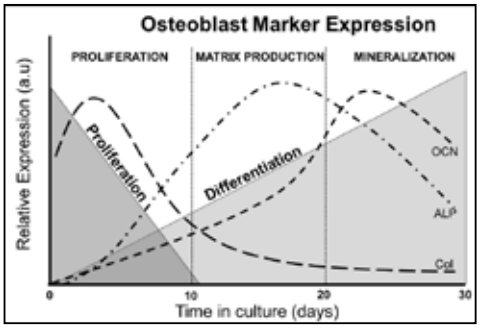

Fig.5. Reciprocal and functionally coupled relationship between cell growth (proliferation, matrix production, and mineralization) and differentiation-related gene expression (ALP, Col, and OC).

3.3 hDPSC-CM increases stained calcium nodules

Calcium nodules stained with red alizarin were more numerous with hDPSC-CM (Figs. 4A, B). The same aspect of DAPI images shows a similar level of cell number in both samples (Figs. 4C, D).

4. DISCUSSION

Many research teams have already studied the influence of conditioned medium on osteoblast differentiation; the majority of them used the CM from bone marrow mesenchymal stem cells (MSCs): only few researchers used the DPSC-CM. Differentiation of MSCs into osteoblasts is studied the most, and differentiation and mineralization need further investigations on true osteoblasts.

Conclusions of previous studies were contradictory: Santos and co-workers[10] (2015) have shown that MSC-CM decreased the proliferation and the differentiation of MSCs[10]. Osugi and associates[11] (2012) and Katagiri and co-workers[13] (2016) have demonstrated that MSC-CM enhances MSC migration, proliferation, and differentiation into osteoblasts; in another study[14], Katagiri and associates (2015) proved that conditioned medium from MSCs enhances early bone regeneration after maxillary sinus floor elevation in rabbits. Sun and associates[15] (2012) showed that MSC-CM transiently retards proliferation and differentiation of primary osteoblasts by downregulating RUNX2 (Runt-related transcription factor 2), a key transcription factor associated with osteoblast differentiation. On the other hand, Fujio and co-workers[16] (2015) have demonstrated that hypoxic DPSC-CM promotes bone healing during distraction osteogenesis, but their findings were just dependent on angiogenesis because they found that hDPSC-CM did not enhance mineralization in vitro.

In the present study, the effect of the conditioned medium derived from human DPSCs was tested on the proliferation and the mineralization of human MG-63 osteoblast-like osteosarcoma cells.

The development of the bone cell phenotype progresses from a proliferating cell to a mature osteocyte in a mineralized type I collagen extracellular matrix. The temporal sequence of osteoblasts gene expression has defined three distinct phases: growth

proliferation, extracellular matrix maturation, and extracellular matrix mineralization[9] (Fig. 5).

Matrix maturation phase is characterized by maximal expression of alkaline phosphatase. ALP is an osteoblast-secreted enzyme present in high levels at sites of bone mineralization; it is involved in the mineralization process by hydrolyzing organic phosphates to release free inorganic phosphate[17] which, after accumulating later with Ca^{2+}, induces the formation of hydroxyapatite (HA)[18].

Furthermore, genes for proteins, such as osteocalcin (OC), bone sialoprotein (BSP), and osteopontin (OPN), are expressed at the beginning of matrix mineralization, and once mineralization is completed, calcium deposition can be visualized using adequate staining methods. Analysis of bone cell-specific markers like ALP, OC, and collagen type I or detection of functional mineralization, is frequently used to characterize osteoblasts in vitro[19].

In the present study, the effect of DPSC-CM was tested on these 3 phases of differentiation of MG-63. Matrix maturation and mineralization were studied by evaluating ALP, non-mineralized collagen type I, and OC amount, and by assessing the calcification with red alizarin staining. The secretion of ALP increases with the effectiveness of hDPSC-CM and matrix is mainly composed of mineralized collagen with smaller but significant amounts of OC. A 50% proportion of hDPSC-CM was chosen as the mean value for testing its effect.

Our results showed that the hDPSC-CM promotes the MG-63 proliferation significantly more than the conventional medium αMEM; its influence on matrix maturation and mineralization is not yet clear. ALP and OC amounts increased with hDPSC-CM and non-mineralized collagen decreased, but non-significantly compared to the control. However, when calcification of matrix was assayed by the measurement of deposited calcium, calcium nodules stained with red alizarin were more numerous with hDPSC-CM. Microscopic images were very clear showing higher frequency of calcium nodules in presence of hDPSC-CM and nearly the same cell number in both samples.

5. CONCLUSION

The role played by paracrine factors produced by stem cells in tissue regeneration and healing has been investigated and many reports have demonstrated that osteogenesis is promoted by conditioned medium (CM) collected from bone marrow mesenchymal stem cells. In this study, we suggest, for the first time, the possible role of hDPSC-CM in the osteogenesis process. Our results indicate that hDPSC-CM promotes osteoblast proliferation. Further studies are warranted in order to confirm its positive effect on bone regeneration.

Conflict of Interest Disclosure Statement

The authors affirm that they have no financial affiliation (employment, honoraria, direct payment, stock ownership, consultantships, patent licensing arrangements) or involvement with any commercial organization or corporation, with any direct financial or economic interest in the subject or materials discussed in this manuscript. Any other potential conflict of interest is disclosed.

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