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3D environment on human mesenchymal stem cells differentiation for bone tissue engineering

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Abstract In this work a novel method was developed to create a three dimensional environment at a cellular level for bone tissue engineering. Biphasic calcium phosphate (BCP) particles of 140–200 μm were used in association with human mesenchymal stem cells (hMSCs). The cells seeded on these particles adhered and proliferated more rapidly in the first day of culture compared to culture on plastic. Analyses of hMSCs cultured without osteogenic factors on BCP particles revealed an abundant extracellular matrix production forming 3-dimensional (3D) hMSCs/BCP particles constructs after few days. Bone morphogenetic 2 (BMP-2), bone sialoprotein (BSP) and ALP gene expression using real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) confirmed that expression profiles were modified by the culture substrate while the addition of osteogenic medium enhanced bone markers expression. These results indicate that BCP particles alone are able to induce an osteoblastic differentiation of hMSCs that might be of interest for bone tissue engineering.

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1 Introduction

Each year, it is estimated that at least 1 million patients need treatment for skeletal disorders. Many of these interventions require bone graft substitutes. The current gold standard used is the autograft because of its osteogenic properties [1]. However, this technique has important limitations such as the need for a second site of surgery, associated morbidity and limited supply. An alternative is the use of biomaterials capable to reproduce bone structure. Various materials are used as synthetic bone substitutes, among which biphasic calcium phosphate (BCP) ceramics are good candidates [2]. These materials mimic bone mineral features (hydroxyapatite) and have proved to be of interest for bone regeneration when associated with human progenitor cells [3, 4].

In recent years, human mesenchymal stem cells (hMSCs) have generated a great interest as potential source for cell based therapeutic strategies. hMSCs represent an adherent, fibroblast-like population [5] present not only in the bone marrow but in a number of tissues, including, blood, adipose tissue, muscle and dermis [6, 7]. Bone marrow derived hMSCs are multipotent cells capable to differentiate into several lineages such as adipocytes, chondrocytes and osteoblasts. An important challenge for bone engineering is the development of treatment strategies for large bone defects [8–10] by association of hMSCs differentiated to osteoblasts and ceramics.

In most cases, ceramics for bone engineering are shaped as porous scaffolds. However, at the cellular level, pores are closer to flat surfaces that do not reproduce the 3D structure of bone native extracellular matrix. This configuration might be important for the cellular organization after implantation and the resulting bone formation. The aim of this study was to create a three dimensional (3D)

ceramic-based culture system for hMSCs for bone engineering. Additionally, the influence of this 3D environment on cell differentiation was investigated. To obtain 3D structures at cellular level, small ceramic particles of BCP were associated with hMSCs, so that they synthesize their own extracellular matrix (ECM) and create structures in 3D. The formation of these 3D constructs was first evaluated macroscopically and cell attachment and proliferation was measured in vitro. The ECM formation was investigated microscopically and the effect of 3D constructs on hMSCs differentiation was monitored and compared to two dimensional (2D) culture using metabolic assays and real time quantitative reverse transcriptase-polymerase chain reaction.

2 Materials and methods

2.1 Ceramic particles

Biphasic calcium phosphate (BCP, 20/80) particles made of hydroxyapatite (20%) and beta-tri-calcium phosphate (80%) with a range size of 140–200 μm (MBCP™) were provided by Biomatlante, Vigneux de Bretagne, France. After processing, the particles were washed in demineralized water and ethanol for 10 min using an ultrasonic bath and finally air dried. The aliquots of BCP particles were double packaged and sterilized in an autoclave at 121°C for 20 min. The chemical purity of the BCP was analyzed using X-ray diffraction (XRD, Philips PW 1830, CuKa source) and Fourier transforms infrared spectroscopy (FTIR, Nicolet, Magna-IR 550). Finally, traces of CaO in the BCP were checked using a phenolphthalein test.

2.2 Cells isolation and expansion

According to the Ethical Committee of Tours University Hospital after written informed consent of three healthy donors, 20 ml of bone marrow were harvested by iliac crest aspiration using Jamshidi needle.

Nucleated bone marrow cells were seeded on polystyrene-treated culture flasks (Corning life sciences, Schiphol-Rijk, The Netherlands) at a density of 5×10^5 cells per cm^2 and human mesenchymal stem cells (hMSCs) were isolated by their adherence capacity. Cells were then amplified in alpha-modified eagle's medium (α-MEM) supplemented with 2 mM L-glutamine, 100 U/ml Penicillin, 100 U/ml Streptomycin, 10% fetal calf serum (FCS, Hyclone) and 1 ng/ml of fibroblast growth factor-2 (FGF-2) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Medium was renewed twice weekly until cells reached confluence. Cells were harvested enzymatically from plastic by an incubation of 3–4 min with 0.25% trypsin/EDTA. To obtain a large number of cells, hMSCs were further expanded on polystyrene-treated culture flasks (Corning life sciences) for further expansion until passage 1. The cells were then harvested and seeded on ceramics particles or plastic dishes.

2.3 Cells culture on BCP particles

To minimize ion release from the biphasic calcium phosphate (BCP) particles during cell culture, particles were incubated 48 h in twice-refreshed proliferative medium (α-MEM, 10% FCS, 1% antibiotic/antimycotic). Prior culture, hMSCs were loaded on BCP particles at a density of 2×10^6 cells per 160 mg. To favor cellular adhesion on ceramic particles, the culture was performed in six low-attachment well plates (Corning life sciences, Schiphol-Rijk, The Netherlands). Cells were then cultured during 1, 4, 7, 14 and 21 days in proliferative medium or in osteogenic medium (proliferative medium supplemented with β-glycerophosphate (sigma G9891, 10 mM), ascorbic acid-2-phosphate (sigma A8960, 0.2 mM), dexamethasone (sigma D4902, 10^{-8} M). The culture mediums were refreshed every 3 days.

2.4 Microscopic evaluation of hMSC/BCP particles constructs

The microstructure of BCP particles after autoclave sterilization and of 3D constructs after culture were observed using a scanning electron microscope (SEM, Leo 1450VP, Zeiss, Germany). Prior to SEM observations, all samples were fixed, dehydrated and coated with gold–palladium at 20 mA for 3 min (EM Scope, UK).

2.5 Alkaline phosphatase staining

To precisely compare culture conditions, human mesenchymal stem cells at a density of 2×10^4 cells/cm^2 were cultured during 21 days on both plastic dishes and ceramic particles (corresponding to 5×10^5 cells/160 mg of particles) in proliferative or osteogenic medium. At the end of the culture time, cells were fixed with an acetone/citrate solution during 1 min, washed with distilled water and incubated in a Fast-violet B solution containing Naphtol during 30 min at room temperature in the dark (Sigma–Aldrich, St Quentin Fallavier, France). Samples were finally rinsed in distilled water during 2 min and observed with a binocular.

2.6 Proliferation

Cells from three different patients ($n = 3$) were cultured up to 21 days in proliferative or osteogenic medium onto BCP

particles or on polystyrene culture-treated 6-well plates (Corning life sciences, Schiphol-Rijk, The Netherlands). To quantitatively measure cell proliferation and evidence differences, the cellular density was reduced to 1×10^4 cells/cm^2 for both conditions. After 1, 4, 7, 14 and 21 days cells/BCP particles constructs were washed with PBS and 2 ml of a 10% Alamar blue solution (BioSource, Caramillo, USA) was added to each sample. After 30 min of incubation (37°C, 5% CO_2) the alamar-containing medium was collected and read on a fluorescence plate reader with excitation filter at 490 nm and emission filter at 595 nm. Samples signal intensities were correlated to a standard curve obtained from increasing densities of hMSCs cultured on 6-well plastic plates. These cells were seeded 12 h prior to the assay to obtain an early metabolic proliferation stage. All samples and standards were assayed in triplicate.

2.7 RNA extraction and real time quantitative RT-PCR

Total RNA was extracted using trizol reagent (Invitrogen, Paisley, UK) according to manufacturer's instructions. Briefly, the 3D constructs (constructed from three different bone marrow aspirates, $n = 3$) were transferred into a 1.5 ml tube and vigorously shaked to lyse the cells. A centrifugation at $10{,}000 \times g$ for 15 min at 4°C in the presence of chloroform was then performed. The aqueous phase was collected, and the RNA precipitated by addition of isopropanol and centrifugation at 7,500 g for 10 min at 4°C. RNA pellets were washed with 75% cold ethanol, dried, reconstituted in sterile water, and quantified by spectrometry.

After desoxyribonuclease I digestion (Invitrogen, Paisley, UK), RNA samples (2 µg) were reverse transcribed using avian myeloblastosis virus-reverse transcriptase (AMV-RT) and random primers in a total volume of 25 µl. Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in the iCycleriQDetection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green detection and Titanium Taq DNA polymerase according to the manufacturer's recommendations. The following temperature profile was used: 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 1 min at 60°C and 30 s at 72°C. Expression of the target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase levels (GAPDH). The sequences of primers are summarized in Table 1. Real time quantitative RT-PCR primers were synthesized by Applied Biosystems Inc (Foster City, USA). The ΔΔCt (cycle threshold) method was used to calculate relative expression levels as previously described [11]. Results are reported as fold change in gene expression relative to basal conditions (hMSCs prior to seeding on plastic or on ceramic particles).

2.8 Statistical analysis

All experiments were repeated a minimum of three times and data are presented as mean values ± standard deviation (SD). Statistical differences among groups were evaluated by one-way ANOVA, and Fisher's post hoc multiple-comparison tests (Tukey's) using significance at a P-value < 0.05.

3 Results

3.1 Macroscopic evaluation of 3D constructs

The seeding of hMSCs on BCP particles (20/80 and 140–200 µm of diameter) (SEM, Fig. 1a) induced the formation of small aggregates immediately after addition of the cells and manual homogenization (swirling). Within one day, aggregates merged to form a skin-like layer containing the particles (Fig. 1b), suggesting a strong cellular adhesion and proliferation.

3.2 Cellular proliferation

To confirm the adhesion and proliferative capacities of hMSCs on BCP ceramic particles, the cell number over time was quantified. Results showed in Fig. 2 indicate that most of the hMSCs were capable to adhere on the particles and proliferate. In proliferation medium, the majority of seeded cells were attached on BCP particles and an

Table 1 Primers used for real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene name	Sequences	Primers
Bone morphogenetic protein 2 (BMP-2)	GCTCTTTCAATGGACGTGTCCC	Forward primer 5′ → 3′ region (720–869)
Bone morphogenetic protein 2 (BMP-2)	AGGACCTGGGGAAGCAGCAA	Reverse primer 3′ → 5′′region (720–869)
Bone sialoprotein (BSP)	GGGCAGTAGTGACTCATCCGAAG	Forward primer 5′ → 3′region (325–533)
Bone sialoprotein (BSP)	CTCCATAGCCCAGTGTTGTAGCAG	Reverse primer 3′ → 5′ region (325–533)
Alkaline phosphatase (ALP)	TCAAGGGTCAGCTCCACCACA	Forward primer 5′ → 3′ region (471–677)
Alkaline phosphatase (ALP)	ATTGGCCTTCACCCCACACA	Reverse primer 3′ → 5′ region (471–677)

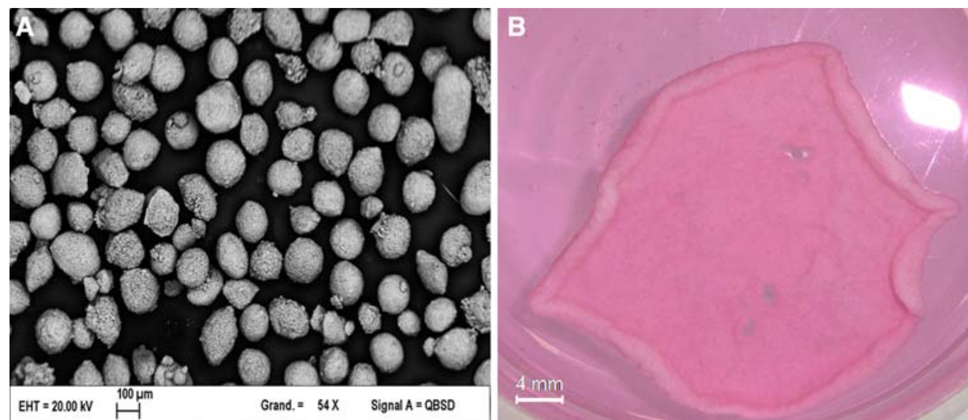

Fig. 1 Formation of 3D constructs. Association of biphasic calcium phosphate particles between 140 and 200 µm (**a**) with human mesenchymal stem cells forms a compact 3D construct (**b**)

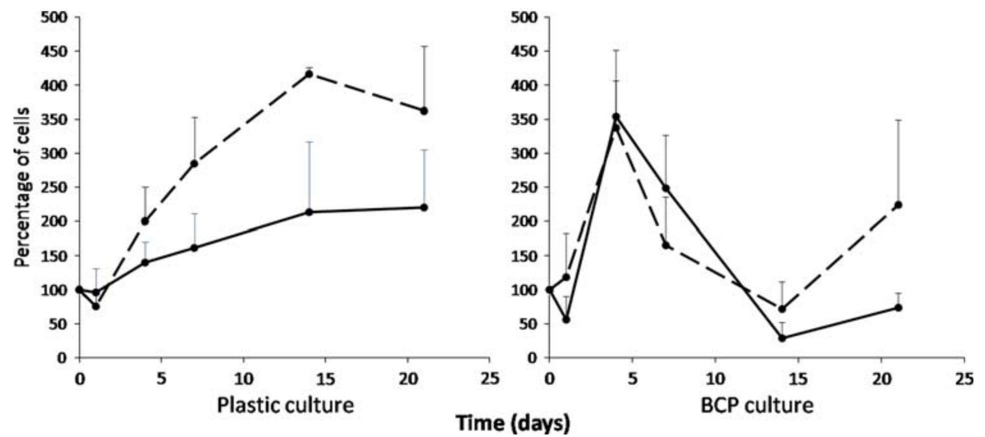

Fig. 2 hMSCs Proliferation on different substrate. A fraction of cells was cultured on polystyrene-treated culture plastic and another was cultured on BCP (20–80) ceramic particles (140–200 µm) in proliferative (*dotted lines*) or osteogenic medium (*full lines*) during 21 days

increase of cell number was measured after 1 day (110% of cells). As a comparison after one day of culture on plastic dishes, only 80% of cells remained in proliferative medium. In osteogenic medium, 60% of the seeded cells were attached on the particles after 1 day, while 90% were present on plastic.

With regard to proliferation, hMSCs number increased on plastic substrate at a constant rate in proliferative medium until 14 days. The cell number remained then constant. In osteogenic medium, hMSCs proliferation rate was less important with only one population doubling in 21 days. On BCP particles, hMSCs proliferated more rapidly during the first days of culture as compared to plastic. However, after 4 days, the number of cells on BCP particles seemed to decrease both in proliferation and osteogenic medium. On plastic and BCP particles, hMSCs proliferated less rapidly in osteogenic medium.

3.3 Microscopic evaluation of 3D constructs

Scanning electron microscopy (Fig. 3a, b) showed an extensive formation of extracellular matrix (ECM) after 14 days on the ceramic particles. This ECM was produced by hMSCs and covered entirely the particles after only 4 days of culture. Then, this strong cellular network remained around the particles for all culture times.

3.4 Alkaline phosphatase expression

Figure 4 shows alkaline phosphatase (ALP) staining realized on hMSCs after 21 days of culture in different conditions. The enzyme was expressed on plastic only for cells cultured in osteogenic medium. However, when cells were cultured on BCP particles, a positive staining appeared in both proliferative and osteogenic medium, with a slightly higher level in osteogenic medium.

3.5 Gene expression profile

The real time quantitative RT-PCR results are shown in Fig. 5. hMSCs cultured in proliferative medium (Fig. 5a) on particles (3D) showed a higher level expression of BMP-2 and BSP genes than on plastic (2D) after 1, 14 and 21 days of culture. ALP expression levels were similar for cells cultured on particles or on plastic but with different profiles over time. Indeed, cells cultured on plastic presented an increase of ALP expression level over 21 days while there was a decrease when cells were cultured on particles.

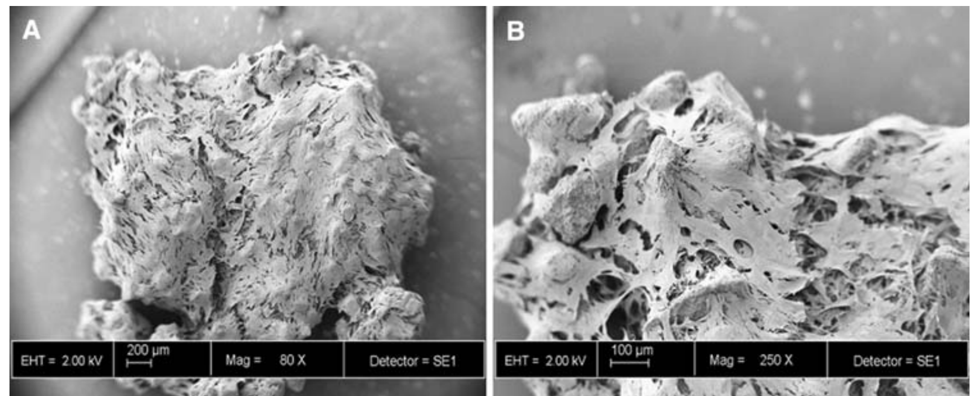

Fig. 3 Scanning electron microscopy of hMSCs/BCP particles construct. Human mesenchymal stem cells were cultured during 14 days on HA-TCP (20–80) BCP particles in proliferative medium. These pictures show the formation of a 3D construct with ECM production by hMSCs at different magnifications: ×80 (**a**) and ×250 (**b**)

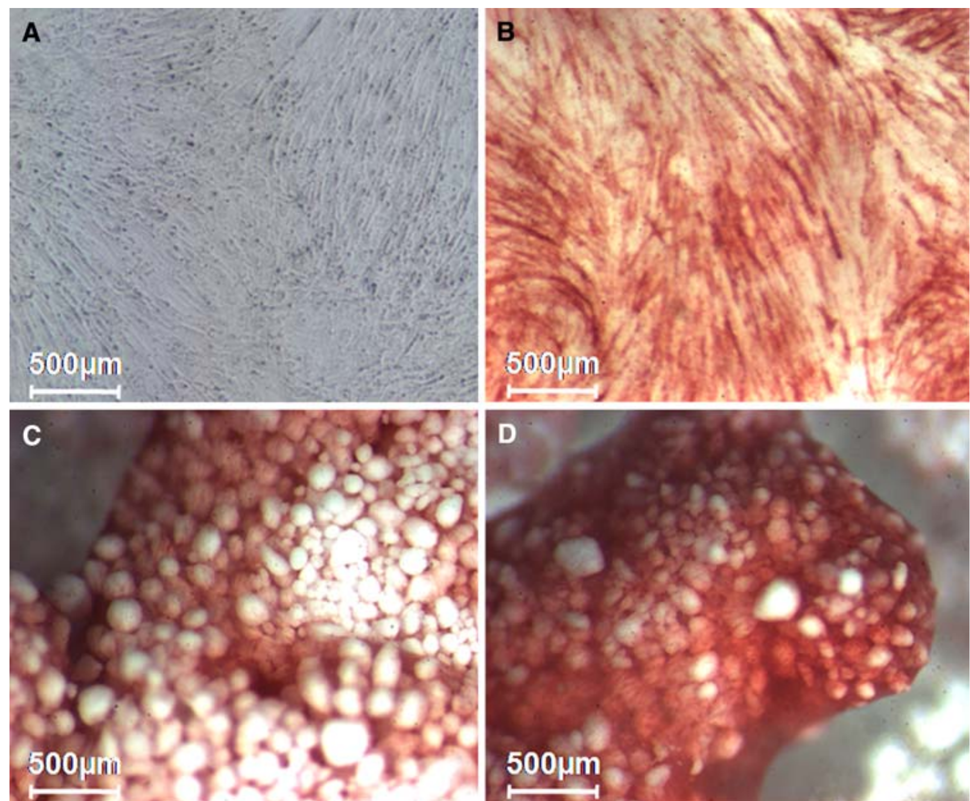

Fig. 4 Alkaline phosphatase staining of human mesenchymal stem cells. A density of 2×10^4 cells/cm^2 are seeded on plastic or on BCP particles and cultured during 21 days in different medium. **a**, **b** hMSCs on plastic culture dishes. **c**, **d** hMSCs on BCP (20/80) particles (140–200 µm). **a**, **c** proliferative medium. **b**, **d** osteogenic medium

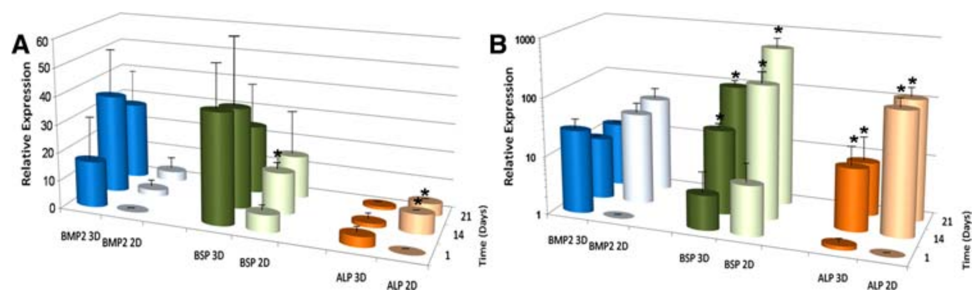

Fig. 5 Influence of 3D (hMSCs/BCP particles constructs) or 2D (plastic) culture on genes expression. Genes relative expressions were measured by real time quantitative RT-PCR SyberGreen. hMSCs were seeded at a density of 2 millions cells/160 mg of particles (140–200 µm) and cultured during 21 days in proliferative (**a**) or osteogenic medium (**b**). * Denotes a significant difference compared to day 1 of the same culture conditions ($P < 0.05$)

With osteogenic medium (Fig 5b) an important increase of genes expression levels was observed compared to proliferative medium. Expressions of BMP-2, BSP and ALP increased widely for hMSCs cultured on plastic (2D). On BCP particles (3D), cellular gene expressions of BSP and ALP were enhanced but not of BMP-2. Moreover, gene expressions profiles of cells cultured on BCP particles were closer to the ones of cells cultured on plastic when osteogenic medium was used instead of proliferative medium. Gene expression of BSP and ALP increased over time for both plastic and particles substrate.

4 Discussion

Within bone tissue engineering, ceramic scaffolds are commonly used to provide a template for cellular colonization and tissue organization. If ceramics are chemically very close to the natural mineral structure of bone, porous scaffolds are very far from bone structure at the cellular level. Instead of a three-dimensional matrix produced by cells and that enclose them, porous scaffolds offer only a flat surface to cells that has been nonetheless proven of interest in various studies [12–14].

Porous scaffolds are then associated with hMSCs that possess valuable proliferation and differentiation capacities [15–17]. To induce bone formation when associated with such scaffolds, extensive investigations have been conducted on the effect of culture medium on hMSCs osteoblastic differentiation [18–21]. As a result, differentiation mediums containing either hormones or growth factors are very often used in conjunction with ceramic porous scaffolds and stromal cells. However, very little interest has been given to the effect of the immediate cell environment on differentiation.

To create a 3D environment based on hydroxyapatite and tri-calcium phosphate ceramic, BCP particles ranging from 140 to 200 μm in diameter were associated with hMSCs. The hypothesis was that cells would adhere on particles surfaces and bridge them during their proliferation, producing so a 3D composite structure based on natural extracellular matrix. Indeed, hMSCs mixed at a specific ratio with BCP particles of a determined diameter range immediately cause their aggregation and form a solid and thick structure rapidly. Quantitative Alamar blue assays confirmed that almost all the seeded cells adhere on the particles after one day of culture. Furthermore, scanning electron microscopy clearly shows that cells cultured on particles produce an abundant extracellular matrix on and between particles. These results indicate the suitability of the particles arrangement as substrate for the creation of a cell-induced 3D environment.

The proliferation capacity of hMSCs within the formed 3D constructs was confirmed over the first four culture days, independently of the culture medium used. At later time points, a decrease of cell number was noticed that could indicate cellular apoptosis. However, the Alamar blue assay is based on cellular metabolic activity. Therefore, a reduction of the assay fluorescence signal can be related to a decline of hMSCs metabolic activity instead of a decrease of cell number, similarly to osteogenic medium that induce a lower proliferation rate of hMSCs cultured on plastic. Such a decline suggests that the 3D environment, provided by extracellular matrix production and BCP particles, induces cellular differentiation.

To confirm that a ceramic-based 3D environment induces the differentiation of hMSCs and to evaluate the differentiation lineage followed, alkaline phosphatase (ALP) expression was investigated. ALP is a key enzyme released from osteoblasts and involved in bone mineralization that locally cleaves phosphates ions from organic phosphate at physiological pH and therefore induces the precipitation of calcium phosphate [22, 23]. Similarly to previous work [24, 25], a positive ALP staining was observed when hMSCs were cultured on BCP particles in osteogenic medium, indicating ALP expression and suggesting osteoblastic differentiation. Surprisingly, ALP expression was also observed when cells were cultured on particles without osteogenic factors. This suggests that the particle-based 3D environment can induce the osteoblastic differentiation of hMSCs. Such an effect could be related to the intrinsic properties of BCP particles or to the effect of the 3D environment produced by the cell. Further experiments will have to determine the relative importance of these parameters on ALP production. However ALP expression alone is not sufficient to confirm hMSCs osteoblastic differentiation induced by osteogenic medium or BCP particles.

Therefore, real time quantitative RT-PCR was performed to obtain an overview of other osteoblastic genes in addition to ALP. In proliferative medium, bone morphogenetic protein 2 (BMP-2), which plays a key role in osteoblasts differentiation, confirms the inductive effect of particles with an increase of mRNA expression compared to plastic culture. A similar result was obtained for bone sialoprotein (BSP), which is secreted by the osteoblasts and could be a nucleator for hydroxyapatite crystals formation [26, 27]. The higher gene expressions observed, as compared to plastic, tend to confirm the osteoblastic differentiation of hMSCs. Surprisingly, ALP gene expression was not related with the enzymatic expression observed.

In osteogenic medium, as expected from previous work [21, 28], gene expressions levels are enhanced on both plastic and particles substrates compared to proliferative medium. Gene expression of osteoblastic markers increased over time, with the same pattern regardless of culture substrate, suggesting that osteogenic medium levels off relative gene expression profile of hMSCs. The

differentiation factors present in osteogenic medium leads to an osteoblastic commitment of hMSCs independently of their environment.

5 Conclusions

Novel three dimensional constructs, associating biphasic calcium phosphate particles and human mesenchymal stem cells, were developed to study the influence of environment on osteoblastic differentiation. This paper demonstrates the capacity of hMSCs to adhere and proliferate on BCP particles and to form 3D constructs easy to handle. Moreover an induction effect of the cells/BCP particles constructs on hMSCs towards the osteoblastic lineage was observed. Although this effect was less pronounced on bone markers gene expression than the effect of osteogenic medium, it might be useful in vivo. Therefore these new 3D constructs appear as good candidates for bone tissue engineering and potentially clinical applications. Further in vivo studies will determine practically the bone formation potential of this scaffold in an ectopic site.

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References

1. Burchardt H. Biology of bone transplantation. Orthop Clin North Am. 1987;18:187.
2. Fischer EM, Layrolle P, Van Blitterswijk CA, De Bruijn JD. Bone formation by mesenchymal progenitor cells cultured on dense and microporous hydroxyapatite particles. Tissue Eng. 2003;9:1179.
3. Kruyt MC, Dhert WJ, Yuan H, Wilson CE, van Blitterswijk CA, Verbout AJ, et al. Bone tissue engineering in a critical size defect compared to ectopic implantations in the goat. J Orthop Res. 2004;22:544.
4. Mankani MH, Kuznetsov SA, Fowler B, Kingman A, Robey PG. In vivo bone formation by human bone marrow stromal cells: effect of carrier particle size and shape. Biotechnol Bioeng. 2001;72:96.
5. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol. 1974;2:83.
6. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143.
7. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276:71.
8. Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH and Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin Orthop Relat Res. 1998;S247.
9. Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, et al. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res. 2000;49:328.
10. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nat Biotechnol. 2000;18:959.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402.
12. Cooper LF, Harris CT, Bruder SP, Kowalski R, Kadiyala S. Incipient analysis of mesenchymal stem-cell-derived osteogenesis. J Dent Res. 2001;80:314.
13. Dennis JE, Haynesworth SE, Young RG, Caplan AI. Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: effect of fibronectin and laminin on cell retention and rate of osteogenic expression. Cell Transplant. 1992;1:23.
14. Phinney DG, Kopen G, Righter W, Webster S, Tremain N, Prockop DJ. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. J Cell Biochem. 1999;75:424.
15. Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells with osteogenic potential from human marrow. Bone. 1992;13:81.
16. Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res. 1997;12:1335.
17. Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet. 1987;20:263.
18. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991;9:641.
19. de Bruijn JD, Van den Brink I, Mendes S, Bovell YP, van Blitterswijk CA. Bone induction by implants coated with cultured osteogenic bone marrow cells. Adv Dent Res. 1999;13:74.
20. Maegawa N, Kawamura K, Hirose M, Yajima H, Takakura Y, Ohgushi H. Enhancement of osteoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2). J Tissue Eng Regen Med. 2007;1:306.
21. Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. 1988;254:317.
22. Lorch IJ. Alkaline phosphatase and the mechanism of ossification. J Bone Joint Surg Am. 1949;31B:94.
23. Kaysinger KK, Ramp WK. Extracellular pH modulates the activity of cultured human osteoblasts. J Cell Biochem. 1998; 68:83.
24. Di Silvio L, Gurav N, Sambrook R. The fundamentals of tissue engineering: new scaffolds. Med J Malays. 2004;59(Suppl B):89.
25. Saldana L, Sanchez-Salcedo S, Izquierdo-Barba I, Bensiamar F, Munuera L, Vallet-Regi M, et al. Calcium phosphate-based particles influence osteogenic maturation of human mesenchymal stem cells. Acta Biomater. 2009;5:1294.
26. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA. 1993;90:8562.
27. Hunter GK, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. Biochem J 1994;302(Pt 1):175.
28. Frank O, Heim M, Jakob M, Barbero A, Schafer D, Bendik I, et al. Real-time quantitative RT-PCR analysis of human bone marrow stromal cells during osteogenic differentiation in vitro. J Cell Biochem. 2002;85:737.