Osteogenic and chondrogenic differentiation: comparison of human and rat bone marrow mesenchymal stem cells cultured into polymeric scaffolds

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Hyaluronan based scaffold were used for *in vitro* commitment of human and rat bone marrow mesenchymal stem cells (MSC). Cells were cultured either in monolayer and in 3D conditions up to 35 days. In order to monitor the differentiating processes molecular biology and morphological studies were performed at different time points. All the reported data supported the evidence that both human and rat MSC grown onto hyaluronan-derived three-dimensional scaffold were able to acquire a unique phenotype of chondrocytes and osteocytes depending on the presence of specific differentiation inducing factors added into the culture medium without significative differences in term of time expression of extracellular matrix proteins.

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Stem cells, essential building blocks of multicellular organisms, are capable of both self-renewal and differentiation into at least one mature cell type. Stem cells are extremely versatile, differentiating as a function of when and where they are produced during development. The best characterized are embryonic stem cells (ESCs) derived from very early embryos. These cells proliferate indefinitely in culture, while retaining the capacity to differentiate into virtually any cell type when the appropriate site of the developing organism is reached. Thus, ESCs can generate large quantities of any desired cell useful for clinical purposes (Jorgensen C, *et al.* 2004). Stem cells collected from adult tissues or older embryos appear more restricted in their developmental potential, their ability to proliferate, and their capacity for self-renewal. Human bone marrow has a multipotent population of cells capable of differentiating into a number of mesodermal lineages. Mesenchymal stem cells (MSCs) are, in fact, the progenitors of all connective tissue cells. MSCs have been successfully isolated from the bone marrow of a variety of species including human, rat; dog; mouse and rabbit (Radice *et al.* 2000). After expansion in culture, they differentiate into several tissues such as bone, cartilage, fat, muscle, tendon, liver, kidney, heart, and even brain cells (Alhadlaq A *et al.* 2004). Due to their multilineage differentiating potential, and to their capacity to undergo extensive replication without losing this capacity, MSCs have enormous potential in the fields of cell therapy and tissue engineering. These cells can be induced to differentiate when submitted to specific environmental factors; however, to regenerate a true functional human tissue for *in vivo* application, it is necessary the use of fully characterized MSC and scaffolds. The behaviour of MSC embedded in biomaterials, in the long term and in the context of pathological joints,

remains to be studied before clinical application can take place. On the light of these considerations in the present study, we compared the differentiation of MSCs collected from two of the most utilized bone marrow species: human and rat.
Using tissue engineering techniques and hyaluronan (HA) derived biopolymers as supporting scaffolds for three dimensional *in vitro* cell culture, MSCs were stimulated to give rise to bone and cartilage tissue. Biopolymers (HYAFF^{tm} biomaterial, Fidia Advanced Biopolimers, Abano Terme, Padova, Italy) have been extensively studied for *in vitro* reconstruction of tissues such as epidermis, dermis and cartilage (Tonello C, *et al.* 2005; Brun *et al.* 1999). These engineered tissues are used in clinical practice for the treatment of skin and cartilage lesions (Galassi *et al.* 2000; Hollander AP, *et al.* 2006).
In the current study, progenitor cells were seeded into an HA biomaterial of non-woven mesh and cultures were supplemented with chondrogenic and osteogenic medium to develop bone and cartilage tissue *in vitro*. Time course of expression for the principal extracellular protein of bone and cartilage were analized and compaired.

Material and methods

Biomaterials

The biomaterial used in the present study was derived from the total esterification of hyaluronan (synthesized from 80-200 kDa sodium hyaluronate) with benzyl alcohol, and is referred to as HYAFF-11®. The final product is an uncrosslinked linear polymer with an undetermined molecular weight; it is insoluble in aqueous solution yet spontaneously hydrolyzes over time, releasing benzyl alcohol and hyaluronan. HYAFF-11® was used to create non-woven meshes of 50 μm-thick fibers, with a specific weight of 100 g/m^2. These devices were obtained from Fidia Advanced Biopolymers (FAB, Abano Terme, Italy).

Flow cytometric analysis

For flow cytometric analysis, the following phycoerythrin (PE)– and fluorescein isothiocyanate (FITC)–labeled mouse monoclonal antibodies and isotype negative controls were used: CD29-PE, CD166-PE, CD14-PE, CD34-PE, CD45-PE, SH2-PE, SH3-FITC, CD73 –PE and SH4–PE (DAKO, Glostrup, Denmark; Beckman Coulter, Miami, FL). Cells were incubated with antibody for 15 minutes at room temperature for labelling, washed twice with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS. Flow cytometric analysis was performed with a FACScan (Becton Dickinson), for which settings and compensation were adjusted weekly by means of CaliBRITE beads (Becton Dickinson). The data were analyzed by CELLQuest and PAINT-A-GATE software (Becton Dickinson).

Cell cultures

Human/Rat Bone Marrow Mesenchyal Stem Cell (MSC) cultures

Bone marrow aspirates from human/inbred Fisher rat (Charles River Laboratories, Wilmington, MA, USA) femur were seeded on Petri dishes. After one day of culture, the medium was discarded and the adherent cell layer was washed twice and then cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. The media were changed twice a week and MSCs were allowed to grow until confluence. Cells were then trypsinized, tested for viability by eosin exclusion dye and finally seeded on HYAFF-11® three-dimensional scaffolds as described below.

Three-dimensional and monolayer cultures

Pieces (1×1 cm) of the HYAFF-11® non-woven material were fixed to culture plates with a fibrin clot and MSCs were seeded at a density of 5×10^5 cells/cm^2. MSC were seed onto Petri dishes (1 cm^2)at the same density. Culture media were supplemented with the following osteoblastic or chondrogenic factors:

Osteoblastic induction

DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 50 μg/mL L-ascorbic acid (Sigma), 10 ng/mL fibroblast growth factor (FGF) (Calbiochem, CA), dexamethasone 10nM; β glycerophosphate 10 mM.

Chondrogenic induction

DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 50 μg/mL L-ascorbic acid (Sigma), 1 ng/mL transforming growth factor-β1 (TGF-β1) (Calbiochem, CA), 1 ng/mL of insulin (Sigma), 1 ng/mL epidermal growth factor (EGF), (Sigma) and 10 ng/mL basic fibroblast growth factor (EGF) (Sigma).

Table 1.

Primer	*Sequence*	*Size*
Human GAPDH	S TGGTATCGTGGAAGGACTCATGAC AS TGCCAGTGAGCTTCCCGTTCAGC	190
Human Osteocalcin	S ATGAGAGCCCTCACACTCCTC AS CTAGACCGGGCCGTAGAAGCG	303
Human Osteonectin	S ACATGGGTGGACACGG AS CCAACAGCCTAATGTGAA	405
Human Osteopontin	S CTTTCCAAAGTCAGCCGTGAATTC AS ACAGGGAGTTTCCATGAAGCCACA	532
Human Coll I	S GGTGGTTATGACTTTGGTTAC AS CAGGCGTGATGGCTTATTTGT	702
Human Coll II	S AACTGGCAAGCAAGGAGACA AS AGTTTCAGGTCTCTGCAGGT	621
Rat GAPDH	S GCCATCAACGACCCCTTCATT AS CGCCTGCTTCACCACCTTCTT	212
Rat Osteocalcin	S CAGCCCCCTACCCAGAT AS TGTGCCGTCCATACTTTC	232
Rat Osteonectin	S ACTGGCTCAAGAACGTCCTG AS GAGAGAATCCGGTACTGTGG	438
Rat Osteopontin	S CCAAGTAAGTCCAACGAAAG AS GGTGATGTCCTCGTCGTCTA	348

Table 2.

Amplification product	*Annealing T°*	*Time*	*Cicle*
Human GAPDH Human Coll I	62° C	60 sec	25
Human Osteocalcin Osteopontin Osteonectin	70 °C	60 sec	40
Human Coll II	65 °C	60 sec	40
Rat GAPDH	58 °C	60 sec	35
Rat Osteocalcin Osteopontina Osteonectina	58 °C	60 sec	40

After 3, 7, 14 and 21 days of culture, scaffolds and supernatants were separately collected and analysed for cell growth and differentiation.

In vitro proliferation of MSC cultures

To determine the kinetics of cell growth in monolayer and three-dimensional cultures, the MTT-based (Thiazolyl blue) cytotoxicity test was performed on days 3, 7, 14 and 21 according to the method of Denizot and Lang (Denizot *et al.* 1986) with minor modifications.

Electron microscopy

For ultrastructural evaluation, at day 21 three-dimensional osteogenic cultures were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 3 h, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in araldite. Semithin sections were stained with toluidine blue and used for light microscopy analysis. Ultrathin sections were stained with uranyl acetate and lead citrate, and analyzed with a Philips EM400 electron microscope.

Immunohistochemical and histological analysis of three-dimensional cultures

Cryostatic sections (7 μm) of three-dimensional HYAFF-11® cultures were layered over gelatine-coated glass slides, fixed with absolute acetone for 10' at room temperature, and cryopreserved at -20°C until use. Type II collagen fibers present in the MSC-secreted extracellular matrix were visualized with the APAAP procedure (acid phosphatase anti-acid phosphatase). Briefly, after saturating non-specific antigen sites with 1:20 rabbit serum in 0,05M maleate TRIZMA (Sigma) pH 7,6 for 20', both 1:100 mouse anti-human/rat type II collagen (Sigma) were added to the samples. After incubation, samples were rinsed with buffer solution, and then second antibody was added for 30' (Link Ab-DAKO-, rabbit anti-mouse). After rinsing, sections were incubated for 30' with 1:50 mouse APAAP Ab-DAKO, rinsed again, and lastly, reacted for 20' with the Fast Red Substrate (Sigma). Counter staining was performed with haematoxylin (Sigma).

Real time RT-PCR

For each target gene, primers and probes were selected using Primer3 software . All primers are listed in Table 1. Gene expression was measured using real-time quantitative PCR on a Rotor-GeneTM 3500 (Corbett Research). PCR reactions were carried out using the primers at 300 nM and the SYBR Green I (Invitrogen) (using 2 mM MgCl2) with 40 cycles of 15 s at 95°C and 1 min at 60°C. All cDNA samples were analysed in duplicate. Fluorescence thresholds (Ct) were determined automatically by the software with efficiencies of amplification for the studied genes ranging between 92% and 110%. For each cDNA sample, the Ct value of the reference gene L30 was subtracted from the Ct value of the target sequence to obtain the ÄCt. The level of expression was then calculated as 2-ΔCt and expressed as the mean±SD of quadruplicate samples of two separate. Relative

quantitation of marker gene expression (Table 1) is given as a percentage of the beta actin product and the t-test was applied.

Statistical Analysis

The one-way analysis of variance (Anova test) of the software package Excel (Microsoft office 2000) was used for data analyses. Repeat measurement analysis of variance (Re-ANOVA) and paired t tests were used to determine if there were significant ($p<0.05$) changes. Repeatability was calculated as the standard deviation of the difference between measurements of the test performed.

Results

Phenotypic characterization of human MSCs

Figure 1 illustrates the phenotypic characterization of culture-expanded human MSCs (hMSC) by flow cytometric analysis. Cells were consistently positive for β1 integrin (CD 29: 98,98%), CD 166 (95,86%), SH2 (93.22%), SH3 (96,63%) and SH4 (89,35%). Specific hematopoietic markers such as CD 14, CD 34 and CD 45 were consistently negative. Rat MSCs had a similar flow cytometric profile as humans: positivity for CD29; CD166; CD73 (*data not shown*)

MSC proliferation analysis

Figure 2 illustrates MSC growth in the presence of osteogenic differentiating medium in monolayer and three-dimensional conditions. Figure 2a shows that human cells proliferated and peaked as early as day 7. From day 14 to day 21, proliferation decreased and then stabilized at a lower plateau. Rat MSC proliferation peaked at day 15 of culture (Figure 2b). Comparing monolayer with 3D conditions is well evident, for both cell type, the positive effect of non woven on cell proliferation. The maintenance and proliferation of human and rat MSC onto the scaffold is confirmed by the higher MTT values. Indeed, in monolayer cells reach in 15 days confluence conditions showing a plateau in MTT value lower than 3D one where cells are able to growth in a bigger substrate eluding contact inhibition effect.

In Figure 3, the proliferation profile of human/rat MSCs cultured with chondrogenic differentiating medium is reported. Figure 3a illustrates human MSCs that had proliferated in three-dimensional

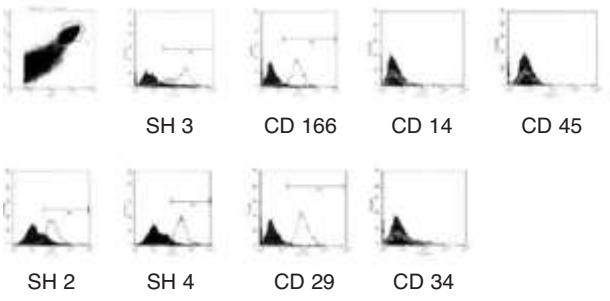

Figure 1. Cytofluorometric analysis of CD 29; CD 166; SH2; SH3; SH4; CD 14; CD 34; and CD 45. Solid profile represents cells stained with secondary antibody alone; Open profile represents cells stained with the anti CD 29; CD 166; SH2; SH3; SH4; CD 14; CD 34; and CD 45 antibody. Fibroblast are used as negative controls (*data not shown*). In each panel, the ordinate represents the number of cells. Data from an experiment representative of at least two similar experiments are shown.

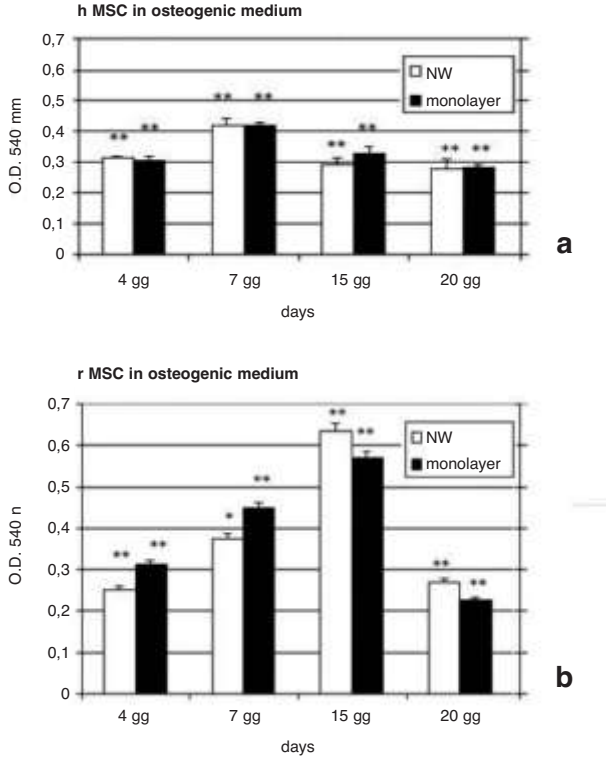

Figure 2. Proliferation rate of human (a) and rat (b) cultured in HYAFF11 ™ non-woven meshes (white bars, NW: non-woven) and in monolayer condition (black bars) in presence of osteogenic medium. The graphs represent the mean of three different experiments. Anova test: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

and monolayer conditions, demonstrating the higher proliferation rate achieved in three-dimensional conditions, particularly in the latest stages of culture. A similar trend was observed in rat MSCs (Figure 3b), although the difference between three-dimensional and monolayer culture conditions was less evident than in human cells.

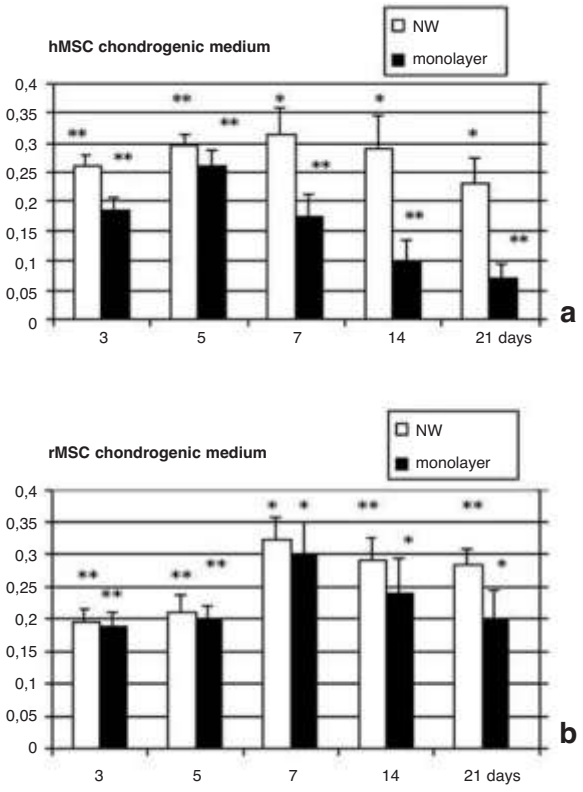

Figure 3. Proliferation rate of human (a) and rat (b) cultured in HYAFF11™ non-woven meshes (white bars, NW: non-vowen) and in monolayer condition (black bars) in presence of chondrogenic medium. The graphs represent the mean of three different experiments. Anova test: *p<0.05; **p<0.01; ***p<0.001.

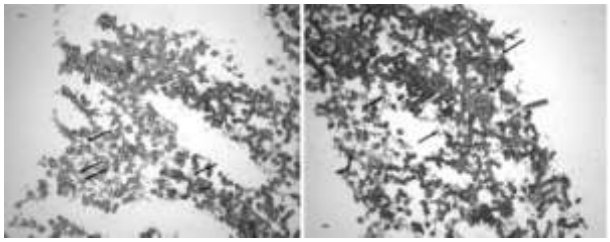

Figure 4. Immunolocalization of type-II collagen in cryostatic section of human (a) and rat (b) MSC after 21 days of culture in 3D cultures in presence of chondrogenic medium. Collagen (black arrows) was present both within the biomaterial interstices and around the biomaterial fibers (white arrows) (X20). Barr: 50 µm

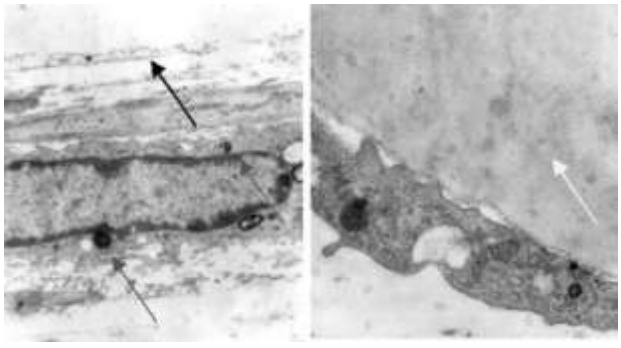

Figure 5. Electron microscopy of hMSC cultured on Hyaff® 11 for 21 days. Cells cultured in osteogenic medium. Some matrix vesicles (grey arrows) are visible in the extracellular matrix close to partially calcified collagen fibres (black arrow). Biomaterials fibers are indicated by yellow arrow. Magnification: (a)= 4600.

Histological and immunohistochemical analysis

Chondrocyte differentiation

Figure 4 illustrates the immunostaining of collagen type II secreted in three-dimensional cultures of both human (Figure 4a) and rat (Figure 4b) MSCs after 21 days. Collagen fibres (black arrows) were present inside the scaffold interstices and the cells filled the inner non-woven fibers (white arrows). A very faint immunostaining reaction for type II collagen was detectable in cells cultured in monolayer (*data not shown*).

Electron microscopy analysis

Electron microscopic analysis of human MSCs in three-dimensional culture (Figure 5) revealed a typical osteoblastic phenotype: a large ovoid nucleus and extensive granular endoplasmic reticulum. Figure 5 a/b illustrates a mineralized area with matrix vesicles in the extracellular spaces close to partly calcified collagen fibres. These cells, which contained a large amount of granular endoplasmic reticulum, were completely surrounded by fully mineralized bone matrix. No significant differences were found between human and rat MSC cultures.

Real time rtPCR

rT-PCR was performed on MSC cultures in monolayer and three-dimensional scaffolds to monitor at the mRNA level cell differentiation in the presence of chondrogenic/osteoblastic medium. Total RNA samples were extracted after 7, 14, 21, 28, 35 days of culture and the expression of chondrogenic/osteoblastic marker genes such as type I and II collagen, osteopontin, osteocalcin, osteonectin was determined. Values are reported as gene/β actin level.

Chondrocyte differentiation

As reported in Figure 6a, collagen type I expression in human and rat MSCs in three-dimensional scaffolds showed a progressive decrease over time. Conversely, collagen type II (Figure 6b) progressively increased in both cell types, peaking at day 21. In monolayer culture of both cell types,

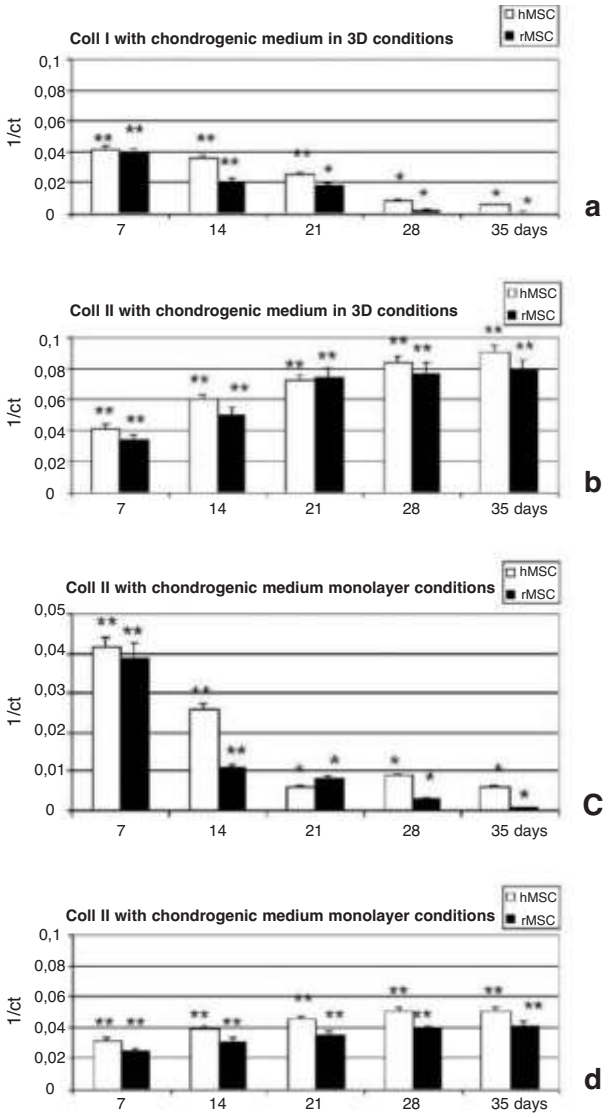

Figure 6. Time course of: collagen I mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (c) in presence of chondrogenic medium. Collagen II mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (b) and in monolayer condition (d) chondrogenic medium.

collagen type I was consistently expressed over time (Figure 6c), while type II collagen was weakly expressed (Figure 6d) and tended to decrease over time.

Osteocyte differentiation

Figure 7a illustrates the expression of collagen type I in human and rat MSCs cultured in three-dimensional scaffolds. Collagen I mRNA production peaked at day 14 and after a temporary drop off at day 21, progressively increased. Figure 7b illustrates the comparatively lower expression of collagen type I in human and rat MSCs cultured in monolayer conditions.

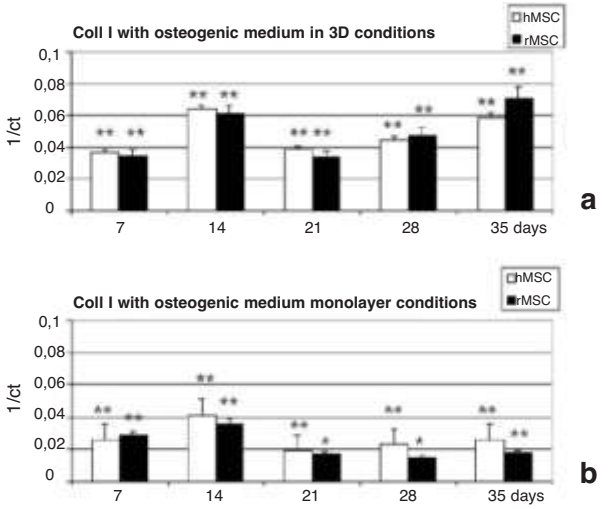

Figure 7. Time course of: collagen I mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of osteogenic medium.

Figure 8a illustrates the expression of osteocalcin, Figure 9a of osteopontin and Figure 10a of osteonectin in human and rat MSCs both in three-dimensional and in monolayer conditions. Osteocalcin expression was similar in both cell types and increase over time. Osteopontin expression was greater than osteocalcin during and appeared constant over time. Osteonectin expression showed a progressive decrease over time for both cell types. In monolayer culture, osteocalcin, osteopontin and osteonectin expression was comparatively lower, but demonstrated the same trend as in three-dimensional cultures (Figures 8/9/10b).

Discussion

In vitro tissue replacement of bone and cartilage has long been a conundrum to be solved by clinicians and tissue engineers. Developments in therapeutic strategies on cartilage repair have increasingly focused on the promising technology of cell-assisted repair proposing to used autologous chondrocytes or other cell types to regenerate articular cartilage in situ. The necessary requisites include the correct cell type and ideal degradable and biocompatible 3D scaffold with favourable structural features for cell attachment, proliferation, chondrogenesis and osteogenesis *in vitro* and functional integration *in vivo*. As regard to biomaterial, hyaluronan based scaffolds, such as HYAFF11, are biodegradable materials currently used for tissue engineering of skin and cartilage. This

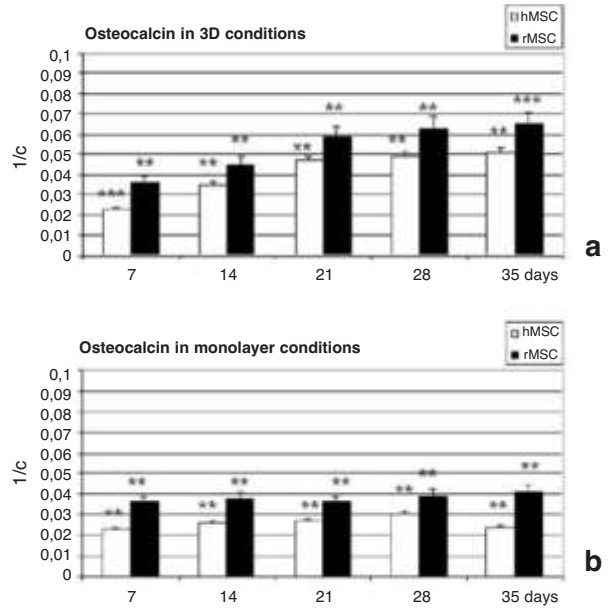

Figure 8. Time course of: Osteocalcin mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of chondrogenic medium.

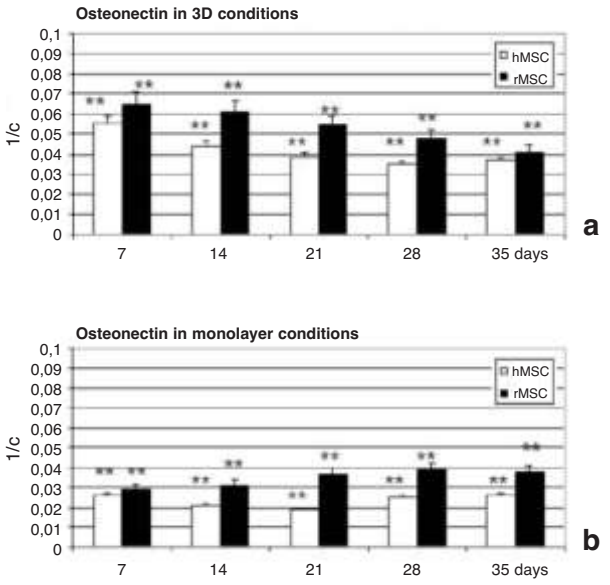

Figure 10. Time course of: Osteopontin mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of chondrogenic medium.

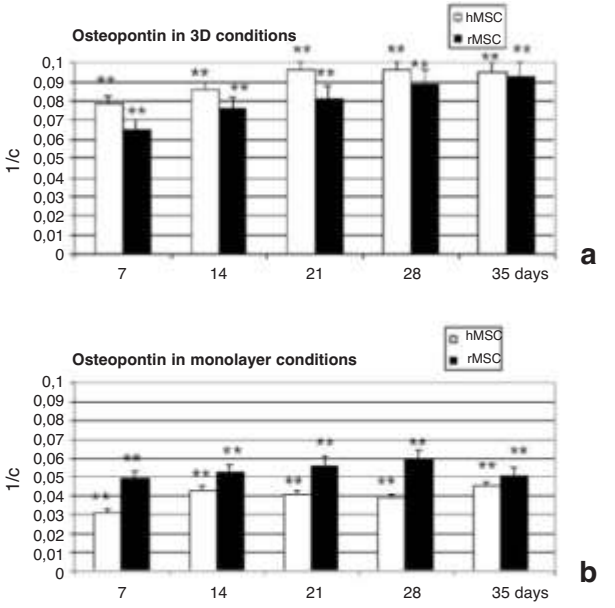

Figure 9. Time course of: Osteopontin mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of chondrogenic medium.

material is highly compatible with cells and matrix and its degradation products induce extracellular matrix production and neoformation of blood capillaries (Tonello *et al.* 2005).

In autologous cell implantation a currently practiced cell-based therapy to repair cartilage defects, autologous chondrocytes are recovered from the patient but are considered too sparse for direct reimplantation. To overcome cell scarcity, chondrocytes are amplified in tissue culture prior to reimplantation, but after at least four doublings, chondrocytes can non longer produce cartilage matrix. In contrast to adult chondrocytes, MSC are easier to obtain and can be manipulated for multiple passages. MSC-based cartilage repair had been attempted in animal models but is still at the early stage of clinical trial for applications in human. MSCs are currently the most promising source for *in vitro* and *in vivo* reconstruction of new hard connective tissue such as bone and cartilage. Indeed, the presently reported data confirm that bone marrow MSCs can be isolated and cultured both in monolayer and in three-dimensional conditions in the presence of chondrogenic/osteogenic medium. Cytofluorimetry confirmed that isolated MSCs from human and rat bone marrow are natural progenitors since they possess the most common specific markers. From the analysis of the principal surface antigens, cells appeared consistently non-hematopoietic and non-endothelial since they were negative for the hallmark antigens of the hematopoietic stem cell such as CD14, CD45, CD34 (Gronthos S, *et al.* 2003). Conversely, they expressed the typical mesenchymal cell markers such as CD29 (anti β1 integrin), SH-2 (recognizing the transmembrane glycoprotein endoglin: CD 105), SH-3 and SH-4 (recog-

nizing CD73) for hMSC and CD73 for rMSC (Haynesworth SE, *et al.* 1992). After expansion in monolayer culture and in the presence of chondrogenic and osteogenic inducing factors, human and rat MSCs differentiated into chondrocytes and osteoblasts, respectively. When cultured in osteogenic conditions, the proliferation rate of MSCs increased during the initial period of culture, progressively decreasing after differentiation both in 3D and in monolayer conditons. Detailed rtPCR analyses of extracellular matrix components (collagen type I, osteopontin, osteocalcin and osteonectin) confirmed the presence of osteogenic molecules already after one week of monolayer or three-dimensional culture. In particular, in this early phase of osteogenic differentiation high levels of osteonectin, a molecule fundamentally important for cellular-bone matrix interaction and for matrix mineralization, were observed in 3D conditions. Collagen type I molecules, essential for formation and maturation of hydroxyapatite crystals, were also detected during the first 10 days of culture. Light and electron microscopy of three-dimensional cultures of MSCs in osteogenic medium demonstrated a well organized extracellular matrix in which type I collagen fibres and calcium phosphate crystals were co-localized. Interestingly, both cell proliferation and expression of human and rat MSCs were consistently higher in osteogenic cells in three-dimensional versus monolayer culture. The three-dimensional hyaluronan scaffolds permitted differentiation of MSCs to a chondrogenic phenotype as well. Time dependent increases in cell proliferation were greater in three-dimensional compared to monolayer culture conditions. These are similar findings to those observed with adult chondrocytes (Brun *et al.* 1999). The expression and production of collagen type II, a well-documented marker of hyaline articular cartilage always found in freshly isolated chondrocytes, was determined by molecular expression and (rtPCR) morphological analyses. Findings again confirmed that the chondrogenic differentiation process was better promoted in three-dimensional culture than in monolayer. Conversely, collagen type I was expressed in three-dimensional culture predominately during the initial phases of the differentiating process, while in monolayer conditions it increased progressively over time. Although human and rat MSCs have the same diferentiating potential, they do behave differently during the proliferation process. While human cell proliferation peaks after one week of culture, rat cell proliferation peaks after two weeks. These results demonstrate that both human and rat MSCs can be cultured in three-dimensional scaffolds made from hyaluronan based polymers in the presence of the necessary stimuli that support differentiation towards osteogenic or chondrogenic phenotypes. The delivery vehicles investigated in this study are easily applicable to clinical practice since hyaluronan scaffolds have been already extensively studied both for the *in vitro* reconstruction of skin and cartilage substitutes and for their clinical application. In the end, these data clearly confirm that bone marrow cells are progenitor cells that are clearly superior to tissue biopsy-isolated cells for use in tissue engineering. Tissue samples from patients have to be isolated by enzymes such as collagenase and hyaluronidase to remove extracellular matrix components and, as is well known, adult stem cells usually are very scarcely supplied within tissues. MSCs isolated from the bone marrow would be a valuable source for cell transplantation since their characteristic features include a high potential for proliferation and multilineage differentiation.

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