Adult mesenchymal stem cells for bone and cartilage engineering: effect of scaffold materials

A. Gigante,[1,2] S. Manzotti,[2] C. Bevilacqua,[1] M. Orciani,[3] R. Di Primio,[3] M. Mattioli-Belmonte[3]
[1]Clinica Ortopedica, Facoltà di Medicina e Chirurgia, Università Politecnica delle Marche, Ancona; [2]Dipartimento di Patologia Molecolare e Terapie Innovative Facoltà di Medicina e Chirurgia, Università Politecnica delle Marche, Torrette, Ancona; [3]Dipartimento di Patologia Molecolare e Terapie Innovative - Istologia, Facoltà di Medicina e Chirurgia, Università Politecnica delle Marche, Torrette, Ancona, Italy

Bone marrow is a useful cell source for skeletal tissue engineering approaches. *In vitro* differentiation of marrow mesenchymal stem cells (MSCs) to chondrocytes or osteoblasts can be induced by the addition of specific growth factors to the medium. The present study evaluated the behaviour of human MSCs cultured on various scaffolds to determine whether their differentiation can be induced by cell-matrix interactions.
MSCs from bone marrow collected from the acetabulum during hip arthroplasty procedures were isolated by cell sorting, expanded and characterised by a flow cytometry system. Cells were grown on three different scaffolds (type I collagen, type I + II collagen and type I collagen + hydroxyapatite membranes) and analysed by histochemistry, immunohistochemistry and spectrophotometry (cell proliferation, alkaline phosphatase activity) at 15 and 30 days.
Widely variable cell adhesion and proliferation was observed on the three scaffolds. MSCs grown on type I+II collagen differentiated to cells expressing chondrocyte markers, while those grown on type I collagen + hydroxyapatite differentiated into osteoblast-like cells.
The study highlighted that human MSCs grown on different scaffold matrices may display different behaviours in terms of cell proliferation and phenotype expression without growth factor supplementation.

Key words: cartilage, bone, tissue engineering, human mesenchymal stem cell, collagen.

Correspondence: Monica Mattioli-Belmonte,
Dipartimento di Patologia Molecolare,
e Terapie Innovative-Istologia,
Università Politecnica delle Marche,
Via Tronto 10/A 60020 Ancona, Italy.
Tel.: +39.071.2206 077.
Tel.: +39.071.2206 073.
E-mail: m.mattioli@univpm.it

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Tissue engineering is one of the most promising therapeutic approaches to bone and cartilage repair. Tissue engineering techniques allow to repair or replace damaged skeletal tissue with autologous or allogenic cells. Despite the positive results obtained in the clinical treatment of cartilage lesions (Peterson *et al.,* 2000), the use of terminally differentiated cells is hampered by the limited sources of normal tissue for harvesting, donor site morbidity, the poor duplicative capacity of chondrocytes and their trend to de-differentiate *in vitro* (Elisseeff, 2004). Treatment of bone defects with autologous bone grafts is associated with morbidity (pain, blood loss, surgical scars), besides the possibility of insufficient graft material (Younger and Chapman, 1989).

Stem cells are a particularly attractive cell type for tissue engineering applications. Their unique high self-renewal activity and multilineage differentiation potential make them an ideal source for cell therapy and regenerative medicine. Adult marrow mesenchymal stem cells (MSCs) (Caplan, 1994) are multipotent cells that are able to generate cartilage, bone and fat (Pittinger *et al.,* 1999; Prockop, 1997). MSCs are easily isolated from human bone marrow (BM) and expanded in culture (Bruder *et al.,* 1997; Gerson, 1999); BM is therefore a good source of cells for skeletal tissue engineering (Kassem and Abdallah, 2008). MSCs do not differentiate spontaneously in culture (Pittinger *et al.,* 1999) nor do they have a progressive commitment towards the osteogenic lineage (Banfi *et al.,* 2002). Their differentiation to tissue-specific cells requires the addition of induction agents and/or appropriate culture conditions (Trubiani *et al.,* 2005; Reyes *et al.,* 2001; Wang *et al.,* 2007; Yoo *et al.,* 1998). Chondroblasts and osteoblasts have been obtained *in vitro* by use of growth factors like basic fibroblast growth factors (bFGF), transforming growth factor-beta (TGF-β)

and bone morphogenetic protein-2 (BMP-2) (Kipnes *et al.,* 2003; Mastrogiacomo *et al.,* 2001; Pittinger *et al.,* 1999).

According to a relatively novel tissue-engineering concept, scaffolds can be designed in such a way as to induce stem cell (or precursor cell) differentiation to a specific phenotype at the implantation site. Cells can therefore be expanded *in vitro* and differentiated into diverse cell types through the action of biomaterial properties such as chemistry, surface energy, wettability, roughness, and topography (Chai and Leong, 2007).

The demonstration that cell-matrix interactions exert a major influence on the behaviour of differentiated cells for tissue engineering (Datta *et al.,* 2005; Gigante *et al.,* 2003) and that surface chemistry is capable of modulating MSC behaviour (Landi and Tampieri, 2006; Mattioli-Belmonte *et al.,* 2005) prompted us to investigate to what extent cell-matrix interactions influence the *in vitro* behaviour of human MSCs cultured on different scaffold matrices.

Materials and Methods

Mcs isolation and characterisation

BM was collected from acetabulum and femoral head of 6 patients (mean age 59 years) undergoing total hip arthroplasty, who gave their informed consent. BM mononuclear cells were obtained by Histopaque® 1077 (Sigma Aldrich, MO, USA) density gradient centrifugation. Cells were plated in 25 cm^2 culture flasks (Falcon®, Becton Dickinson Labware, NJ, USA) in MesenCult™ Basal Medium supplemented with Mesenchymal Stem Cell Stimulatory Supplements (StemCell Technologies Inc., BC, Canada) and 1% penicillin-streptomycin (Gibco Invitrogen, Scotland, UK) for 14 days. Near-confluence cultures were then trypsinised (Trypsin-EDTA 1X -Gibco Invitrogen), expanded (split ratio 1:3) through sequential passages and finally used at the 5^{th} passage.

Cells were characterised using the FACSCalibur flow cytometry system (Becton Dickinson, CA, USA) in line with International Society for Cellular Therapy guidelines (Dominici *et al.,* 2006) using antibodies against the following surface antigens: HLA-DR, CD34, CD105, CD14, CD19 and CD45 (Diaclone, France), CD73 and CD90 (StemCell Technologies Inc.).

Cell cultures on membranes

Three commercial collagen membranes, type I collagen (group A), type I + type II collagen (group B) and type I collagen + hydroxyapatite (HA) (group C) manufactured by Opocrin SpA, Italy) were used.

Cells were seeded on the membranes at an initial density of $2\times10^4/cm^2$ and cultured in 24-well plastic culture plates (Nunc A/S, Denmark) for 30 days. Cells grown on polystyrene served as the control group. Cultures were grown in Dulbecco Modified Essential Medium (DMEM), 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Gibco Invitrogen), in 5% CO2 atmosphere. The medium was replaced at 48-h intervals.

Cell proliferation and alkaline-phosphatase (ALP) production were assayed spectrophotometrically at 15 and 30 days. Histochemical and immunohistochemical analyses were performed at 30 days to study phenotype expression and cell adhesion.

Cell growth

Cell proliferation was evaluated 15 and 30 days into culture by measuring the number of viable cells using the MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) colorimetric assay. In brief, the medium was removed, 200 μL MTT (SIGMA M56655) solution (5 mg/mL in DMEM without phenol red) and 1.8 mL DMEM without phenol red was added to the monolayers; the multi-well plates were incubated at 37°C for a further 4h. After discarding the supernatants, the dark blue formazan crystals were dissolved by adding 2 mL of solvent (0.1N HCl in absolute isopropanol) and quantified spectrophotometrically (UV/Vis Lambda 3 Perkin Elmer, MA, USA) at 570 nm. The results are reported as the percentage of viable cells compared with control cultures (100%). Experiments were performed in triplicate.

Alkaline phosphatase activity

Alkaline phosphatase ALP production was measured at 15 and 30 days on cell lysates with 104 kit (Sigma-Aldrich, Italy) according to the manufacturer's recommendations. Values were calculated from optical absorbance at 420 nm and expressed as international units of enzyme activity per ml (IU/mL). Determinations were performed in triplicate.

Histochemistry and immunohistochemistry

Samples were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, at 4°C for 20 min and washed three times with phosphate-buffered saline (PBS). Histochemical analysis was performed with safranin O, ALP and von Kossa stains.

For immunohistochemistry, non-specific binding was blocked with 3% normal goat serum in PBS, pH 7.4, for 30 min at room temperature; slides were then incubated overnight with primary antibodies at 4°C. Sections were incubated with polyclonal anti S-100 protein (DakoCytomation Denmark A/S, Glostrup DK) diluted 1:3000; anti-collagen type I antibodies (Monosan, The Netherlands) at 1:150; monoclonal anti-chondroitin sulphate (chondroitin-S) antibodies (Sigma Aldrich, MO, USA) at 1:200; anti-collagen type II (Oncogene, CA, USA) 10 μg/mL, and anti-osteonectin (Biodesign Int, ME, USA) 10 nM. Rabbit and mouse immunoglobulins at the same dilutions as the primary antibodies were used as controls. After three washes with Tris-HCl (0.05 M, pH 7.6), the immune reaction was visualised with Dako LSAB+ system - HRP (DakoCytomation Denmark A/S, Glostrup DK) and observed with a Leica microscope (Leica Cambridge Ltd., UK) equipped with a digital camera.

Statistical analysis

Means and standard deviation (SD) were obtained from sums of three different experiments. Data were analysed by one-way ANOVA, Bonferroni's test and Student's t test. Statistical significance was tested at $p<0.05$

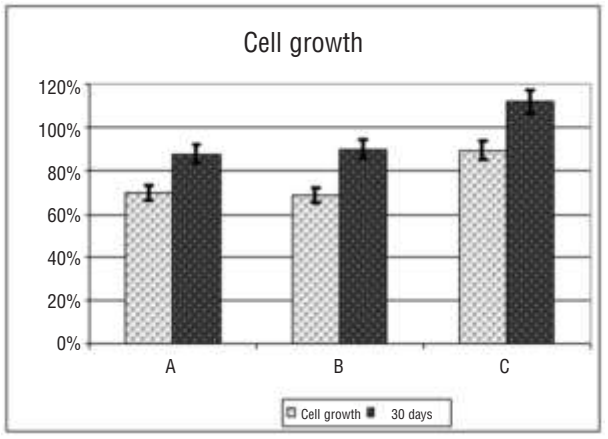

Figure 1. MSC proliferation on different scaffold matrices 15 and 30 days into culture (mean ± SD). Group A: type I collagen membrane; group B: type I + II collagen membrane; group C: type I collagen + HA membrane. Control group: polystyrene.

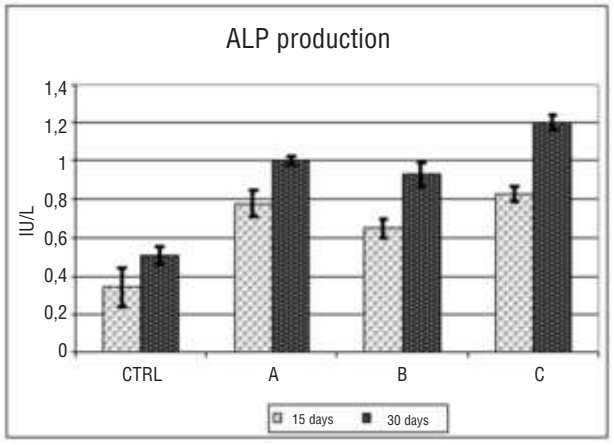

Figure 2. ALP production by MSCs grown on different scaffold matrices at 15 and 30 days. Group A: type I collagen membrane; group B: type I + II collagen membrane; group C: type I collagen + HA membrane. Control group: polystyrene.

Results

Cell growth

The MTT colorimetric assay evidenced an increased number of viable cells with time in all groups (Figure 1). Group C (type I collagen + HA) membranes showed a significant ($p<0.05$) increase in cell proliferation compared with groups A (type I collagen) and B (type I + II collagen) both at 15 and at 30 days.

Alkaline phosphatase activity

A significant ($p<0.05$) increase in ALP activity was detected in groups A and C compared with the control group (Figure 2). In group B differences from the control group were significant ($p<0.05$) only at 30 days. ALP production was highest in group C 30 days into culture.

Histochemistry and immunohistochemistry

Control cells reached confluence and had a polymorphic appearance. Some cells exhibiting a strong ALP reaction were surrounded by numerous unstained cells. Staining for safranin O and von Kossa was seen only in some fields. Type I collagen immunostaining and mild chondroitin-S expression were also observed. S-100 protein positivity was not detected.

Group A cells stained weakly for safranin O and expressed type I collagen and chondroitin-S. The immunohistochemical reaction for S-100 protein was negative.

Group B monolayers displayed a metachromatic reaction to safranin O and clear S-100 protein (Figure 3), type II collagen and chondroitin-S (Figure 4) immunostaining.

In group C von Kossa stain disclosed mineralised matrixand ALP staining was homogeneous. Cells were positive for osteonectin (Figure 5) and type I collagen and weakly positive for chondroitin-S.

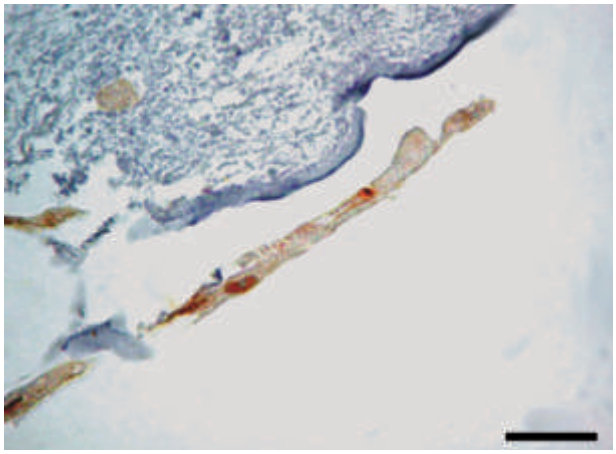

Figure 3. MSCs cultured on type I + II collagen membrane for 30 days. Positive immunoreaction for S-100 protein (scale bar 0.05 mm).

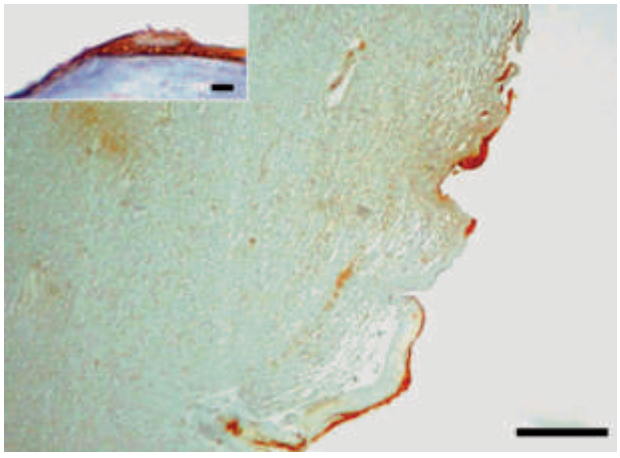

Figure 4. MSCs cultured on type I + II collagen membrane for 30 days. Cells positive for chondroitin-S adhering to the membrane (scale bar 0.1 mm). Inset: positive cells in the scaffold (scale bar 10 um).

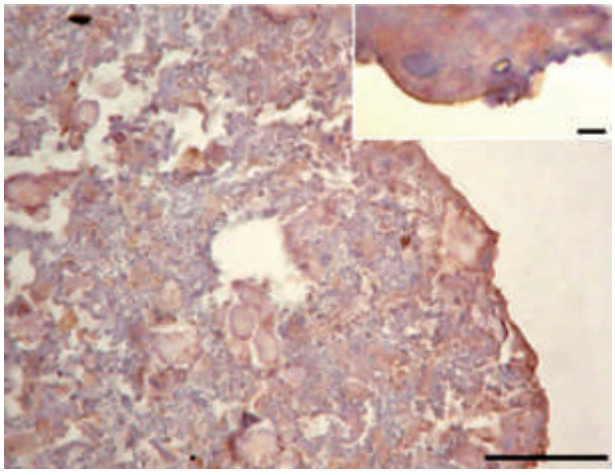

Figure 5. MSCs cultured on type I collagen + HA membrane for 30 days. Positive immunoreaction for osteonectin (scale bar 0,05 mm). Inset high magnification displaying the immunostaining.

Discussion

The use of MSCs for skeletal tissue engineering is based on their high proliferation rate and ability to differentiate to many cell types. Several growth factors, such as bFGF, TGF-β and BMP-2 have been used to induce *in vitro* MSC differentiation (Barry *et al.,* 2001; Johnstone *et al.,* 1998; Kipnes *et al.,* 2003; Mastrogiacomo *et al.,* 2001) Medium composition (serum free vs FBS; low/high-glucose content; addition of β-glycerophosphate and dexamethasone) and culture conditions, including cell density and distribution (monolayer, pellet), have been demonstrated to affect MSC behaviour (Ciapetti *et al.,* 2006; Muraglia *et al.,* 2003; Reyes *et al.,* 2001; Yoo *et al.,* 1998).

Previous research on mature chondrocytes cultured on different collagen membranes has demonstrated that the biochemical composition of the scaffold matrix can influence cell behaviour (Gigante *et al.,* 2003). Cell-matrix interactions may thus be an additional mechanism for modulation of MSC differentiation.

MSC chondrogenic differentiation has been obtained using gelatine-based resorbable sponges (Ponticiello *et al.,* 2000), photopolymerizing polyethylene glycol-based hydrogel (Williams *et al.,* 2003) and PLA/alginate amalgam (Caterson *et al.,* 2002) supplemented with TGF-β1 to the culture medium. MSCs adhering to bioceramic composites differentiated to an osteogenic lineage and achieved bone defect repair in some *in vitro* (Kadiyala *et al.,* 1997; Landi and Tampieri, 2006) and *in vivo* studies (Cancedda *et al.,* 2003; Kadiyala *et al.,* 1997).

Recently, hyaluronan-based scaffolds have been used for *in vitro* commitment of human and rat MSCs; also in this study, however, acquisition of the

specific chondrocytic and osteocytic phenotype depended on specific inducing factors added to culture media (Zavan *et al.,* 2007).

The present study documents that human MSCs grown on different scaffold matrices may be induced to display different behaviours in terms of cell proliferation and phenotype expression, without the addition of growth factors to the medium.

Human MSCs cultured on type I+II collagen membranes differentiated to a chondroblastic phenotype, with stronger expression of S-100 protein, chondroitin-S and type II collagen compared with controls and with cells grown on type I collagen. These findings agree with recent data (Bosnakovski *et al.,* 2006), supporting the case for a matrix composed of type II collagen for cartilage tissue engineering.

In contrast MSCs cultured on type I collagen +HA membranes differentiated into an osteoblastic phenotype, demonstrating greater ALP activity and stronger positivity for osteonectin and type I collagen than controls and cells grown on type I+II collagen membranes. This finding may be partially related to the modulation of cell behaviour by substrate stiffness (Mattioli-Belmonte *et al.,* 2008) and is in line with recent data underlying the positive effect of mineral components in inducing MSC differentiation to an osteoblastic phenotype (Bigi *et al.,* 2007; Wang *et al.,* 2007). A scaffold matrix composed of type I collagen and HA, providing support for MSC growth without compromising their osteogenic differentiation ability (Shih *et al.,* 2006), may thus be indicated for bone tissue engineering. These data confirm the scope for cell behaviour control by the substrate and encourage the clinical use of different scaffolds to obtain different skeletal tissues.

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