BONE MARROW CONCENTRATED CELL TRANSPLANTATION: RATIONALE FOR ITS USE IN THE TREATMENT OF HUMAN OSTEOCHONDRAL LESIONS

C. CAVALLO[1], G. DESANDO[2], L. CATTINI[1,2], M. CAVALLO[3], R. BUDA[3], S. GIANNINI[3], A. FACCHINI[1,2,4] and B. GRIGOLO[1,2]

[1]SSD Laboratory RAMSES, Rizzoli Orthopedic Institute, Bologna, Italy; [2]SC Laboratory of Immunorheumatology and Tissue Regeneration, Rizzoli Orthopedic Institute, Bologna Italy; [3]SC I Orthopedic and Traumatologic Clinic, Rizzoli Orthopedic Institute, Bologna Italy; [4]Department of Clinical Medicine, University of Bologna, Bologna, Italy

Received September 3, 2012 – Accepted November 30, 2013

Bone marrow is one of the best characterized stem cell microenvironments that contains Mesenchymal Stem Cells (MSCs), a rare population of non-hematopoietic stromal cells. MSCs have been indicated as a new option for regenerative medicine because of their ability to differentiate into various lineages such as bone, cartilage and adipose tissue. However, isolation procedures are crucial for the functional activity of the transplanted cells. The use of concentrated bone marrow cells (BMCs) enables a cell population surrounded by its microenvironment (niche) to be implanted while avoiding all the complications related to the *in vitro* culture. The cells of the niche are able to regulate stem cell behavior through direct physical contact and secreting paracrine factors. The aim of this study was to characterize BMCs *in vitro* to evaluate their ability to differentiate toward mature cells and try to understand whether there are differences in the chondrogenic and osteogenic potential of cells from patients of different ages. Mononuclear Cells (MNCs) isolated by Ficoll were used as control. Both cell populations were grown in monolayers and differentiated with specific factors and analyzed by histological and molecular biology assays to evaluate the expression of some specific extracellular matrix molecules. The present investigations revealed the ability of BMCs to act as isolated cells. They are able to form colonies and differentiate toward chondrogenic and osteogenic lineages, the latter pathway appearing to be influenced by donor age. The results obtained by this study support the use of BMCs in clinical practice for the repair of osteochondral damage, which might be particularly useful for the "one-step" procedure allowing cells to be directly implanted in operating room.

An important problem in orthopaedics is cartilage tissue lesions after injury or that occurs during osteoarthritic processes (1). Several techniques have been used to treat cartilage lesions, such as debridement (2), drilling subchondral bone (3), microfractures (4), periosteal and perichondral transplantation (5) and, in the last decade, autologous chondrocyte transplantation (6). This therapeutic strategy has been improved by the use of suitable scaffolds, which provide a biodegradable three-dimensional structure for tissue development, thus facilitating also the surgical procedure and the

Key words: bone marrow cells, mesenchymal stem cells, transplantation, osteochondral lesions, differentiation

Mailing address: Brunella Grigolo PhD, SSD RAMSES Laboratory/SC, Immunorheumatology and Tissue Regeneration Laboratory, Istituto di Ricerca Codivilla Putti, Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy
Tel.: +39 051 6366803 Fax: +39 051 6366807
e-mail: brunella.grigolo@ior.it

0393-974X (2013)

expression of specific molecules by the cells (7). The goal is to regenerate a tissue with the structural and mechanical properties of the original one, a result that cannot be obtained by traditional therapeutic approaches. However, the use of mature chondrocytes isolated from articular cartilage and expanded in culture might be displaced by that of Mesenchymal Stem Cells (MSCs), which can be easily isolated from different sources, such as bone marrow (8). The potential of MSCs to produce cartilage and bone-like tissues has been widely shown *in vitro*, in animal and human models (9-11). However, little consensus and in many cases conflicting results regarding the effect of donor age on regenerative potential have been reported (12). A number of authors have reported a decrease in MSCs with increased age and some changes in differentiation, proliferation, attachment, senescence or self-renewal (13), whereas others have not (14). An age-related atrophy of MSCs has been suggested as a cause of decreased number of osteoprogenitor cells and decreased bone formation capacity because of a lack of mature matrix-forming OB (15).

The MSC population contains progenitors capable of *in vitro* proliferation and differentiation in the presence of specific factors such as Transforming Growth Factor-β (TGF-β) and Bone Morphogenic Protein-7 (BMP-7) for cartilage and dexamethasone, β-glycerophasphate (β-GP), and Fibroblast Growth Factor-2 (FGF-2) for bone, which significantly increase the frequency of tissue formation (16). MSCs commit to chondrogenesis and osteogenesis when the proliferation and phenotypic expression of cells are affected and different genes involved in these processes are modulated (17).

Recently, taking into account that MSC activity is due not only to the expression of one or more intrinsic cell capabilities but depends also on the surrounding microenvironment (niche), transplanting concentrated bone marrow cells (BMCs) was hypothesized (18). The concept that a stable, custom microenvironment or niche might control stem cells was initially hypothesized by Schofield (19). In a niche, neighboring subsets of cells and extracellular substrates house stem cells, and provide specialized functions to modulate stem cell self-renewal and progeny production *in vivo* (20). Bone marrow contains not only Hematopoietic Stem Cells (HSCs) and MSCs as a source for regenerating tissues but also accessory cells that support angiogenesis and vasculogenesis by producing several growth factors (21). Following these findings the transplantation of BMCs seems to be an ideal strategy for the regeneration of cartilage lesions that also involve the subchondral bone. Furthermore, the use of total bone marrow might avoid the isolation and expansion phases required to obtain MSCs, the possible malignant transformations of the cells and risks of adverse immune reactions e.g. components of the Foetal Bovine Serum (FBS) added to the culture (22).

To our knowledge, data present in the Literature which are aimed at characterizing bone-marrow-derived cells refer to studies on cells of animal origin which are isolated from bone marrow (23). The aim of the present study was to evaluate human BMCs, obtained by a concentration procedure performed directly in the operating room, by their phenotypical characterization and ability to differentiate towards chondrogenic and osteogenic lineages.

Mononuclear cells (MNCs) from the same patients isolated by synthetic polysaccharid gradient (Ficoll) were used for comparison. Cells were obtained from patients of different age in order to verify the influence of this factor on the differentiation potential.

MATERIALS AND METHODS

Bone marrow harvesting and concentration

Bone marrow was obtained from the iliac crest of 20 patients (mean age 30.35±12.63; 11 women and 9 men) undergoing autologous cell transplantation for the treatment of osteochondral defects. The Ethical Committee of the Institution approved the human protocol for this study. All investigations were conducted in conformity with ethical principles of research, and written informed consent was signed by all the patients enrolled in this research. The bone marrow was harvested from the posterior iliac crest in a sterile regimen, with the patient in prone decubitus and using general or spinal anesthesia. The marrow was aspirated in small fractions from different points to maximize the harvesting of the marrow stromal cells and reduce dilution by peripheral blood. The harvested bone marrow was reduced in volume directly in the operating room, by removing most of the red cells and plasma. Thus, it was possible to obtain a concentrate of nucleated cells, that are stem cells, monocytes, lymphocytes, and other bone marrow

resident cells. The bone marrow was concentrated with a cell separator (Smart PReP®, Harvest Technologies Corp., Plymouth, MA, USA), by a sterile and disposable dedicated kit (BMAC®, Harvest Technologies Corp., Plymouth, MA, USA). Sixty milliliters of bone marrow aspirate was injected into the posterior portion of the double chamber device. This was placed vertically on the rotor of the centrifuge. A 3 min centrifugation at 2500 rpm separated the red cells from the other components with the help of a semi-permeable membrane; then, during a 2 min arrest period, the buffy coat separated from the erythrocytes was transferred into the anterior chamber. Finally, a 9 min phase at 2300 rpm separated the cellular component from the plasma in the anterior chamber; at the end of the entire process the majority of the red cells remained in the posterior chamber of the container and the mononuclear cells were found at the bottom of the anterior chamber of the device, covered by the platelet-poor plasma. The cells were then aspirated with a syringe and re-suspended into platelet-poor plasma up to 6 ml of cell concentrate. Two out of the 6 ml of concentrate was sent to the authors' laboratory for the experiments *in vitro*. 0.5 ml of concentrate was used *in toto* and 1.5 ml was used to isolate mononuclear cells by Ficoll procedure.

MNC isolation

MNCs were isolated from bone marrow concentrate. 1.5 ml of bone marrow was diluted 1:10 with Phosphate Buffer Saline (PBS), collected in 50 ml tubes and isolated by Ficoll gradient (d=1.077 g/ml) (Sigma, St. Louis, MO, USA). The ring was collected and cells were washed twice, re-suspended in alpha-minimal essential medium (α-MEM) (Sigma) with 15% FBS and counted using Turk dye (Farmitalia Carlo Erba, Milano, Italy).

Analyses performed

Cells, both BMCs and MNCs, were analyzed by flow cytometry analysis (FACS) to evaluate their phenotypic expression. *In vitro* self-renewal capacity was demonstrated by Colony-Forming Unit Fibroblast (CFU-F) assays. Moreover, their differentiation capacity toward the osteogenic and chondrogenic lineages was also evaluated by Alcian Blu and Alizarin Red (Sigma) staining and by molecular biology investigations.

Flow cytometric analysis

A total of $10x10^6$ of BMCs and MNCs were used for the evaluation of specific markers by three-colour immunofluorescence. The cells were washed and resuspended with PBS containing 2% FBS and 0.1 % sodium azide (Sigma). Specific binding block was performed by incubating the cells for 10 min at Room Temperature (RT) with immunoglobulins (Dako, Glostrup, Denmark) of the same isotype of the primary antibodies used. $2.5x10^5$ cells for each marker were successively incubated with mouse monoclonal antibody anti-human CD45 conjugated with Tetramethyl Rhodamine Isothiocyanate (TRITC) (Dako), anti-human CD34 conjugated with Phycoerythrin (PE) and anti human CD90, CD105, CD106, CD146 conjugated with Fluorescein Isothiocyanate (FITC) (Serotec, Oxford, UK) at a concentration of 1 μg/ml for each antibody in the dark for 30 min on ice. Then, the cells were washed twice and fixed with 1% paraformaldehyde and analyzed with a FACStar Plus (Becton Dickinson, Mountain View, CA). Negative and isotype-matched controls were performed. The percentages of specific markers were evaluated on CD34 and CD45 negative cells.

Colony-Forming Units-Fibroblast (CFU-F) assay

The clonogenic ability of BMCs and MNCs was determined by a low-density CFU-F assay $5x10^4$ cells from the two different populations were seeded in Petri dishes in α-MEM supplemented with 20% FBS. The medium was changed twice a week. At 7 and 14 days the wells were washed with PBS, and the cells were fixed with methanol and stained with Crystal Violet (Sigma). An aggregate of cells containing more than 50 cells was classified as a colony originating from one clonal cell.

Chondrogenic and osteogenic differentiation of BMCs and MNCs

The differentiation potential of both BMCs and MNCs was performed in monolayers. Despite being aware that chondrogenesis should be performed in 3D, in the present experiments pellet cultures could not be obtained from BMCs. $1.5x10^5$/wells of both BMCs and MNCs were seeded onto a 12-well plate in α-MEM supplemented with 15% FBS. For each sample one plate was used for histochemical assay and one for molecular biology analysis. Different plates were used for the experimental times scheduled. For chondrogenic differentiation the medium was replaced after 24 h with complete medium Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY, USA) with high glucose supplemented with ITS Premix: 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 5.33 μg/mL linoleic acid, 1.25 mg/mL Bovine Serum Albumin (BSA) (BD Biosciences, Bedford, MA), 10^{-7} M dexamethasone (Sigma), 37.5 μg/mL ascorbate-2 phosphate (Sigma), 1 mM of sodium pyruvate (Sigma) and 10 ng/mL of TGF-β1 (R&D Systems).

For osteogenic differentiation the initial medium was substituted with: αMEM (Sigma) with 15% FBS (Cambrex) containing 75 μg/mL ascorbate-2 phosphate (Sigma), 0.01 mM β-GP (Sigma), 10^{-7} M dexamethasone

(Sigma).

Complete medium without the specific growth factors was used for chondrogenic and osteogenic control respectively. Cells (for both chondrogenesis and osteogenesis) were maintained in culture up to 28 days and the medium was changed twice a week.

Alcian Blue staining

To assess chondrogenic differentiation Alcian Blue staining was performed at 21 and 28 days. Briefly, cultured cells were washed with PBS, fixed in 10% neutral buffered formalin for 30 min at RT. Then, the cells were incubated for 3 min at RT with a 3% acetic acid solution and stained with 1% Alcian Blue solution for 30 min at room temperature. Stained cells were washed extensively in running tap water and rinsed in Double Deionizer Water (DDW).

Alizarin Red staining

To assess osteogenic differentiation, the calcium deposition was evaluated using Alizarin Red S staining at 21 and 28 days. Briefly, cultured cells were washed with PBS, fixed in 10% neutral buffered formalin for 1 h at room temperature. After washes in DDW, the cells were dehydrated in alcohol graded series and stained with 1% Alizarin red S solution (Sigma) for 2 min. Stained cells were washed extensively with DDW to remove the non-specific precipitation, positive red staining represents calcium deposits on the differentiated cells. Experiments were performed in duplicate wells.

Analysis of mRNAs expression by Real-Time PCR

BMCs and MNCs were analyzed by Real-Time RT-PCR to investigate the expression of specific markers during chondrogenesis and osteogenesis at 0 and 28 days for the former and at 0, 21 and 28 days for the latter. In particular, collagen type II, aggrecan and Sox-9 were evaluated in chondrogenic cells and Alkaline Phosphatase (AP), Bone Sialoprotein (BSP) and osteocalcin (OC), were evaluated in osteogenic cells. $1.5x10^5$ cultured cells were directly lysed by the addition to each well of 0.5 ml of TRIzol reagent (Invitrogen, Life Technologies). RNA was recovered by precipitation with isopropyl alcohol and then treated with DNase I (DNAfree Kit, Ambion, Austin, TX, USA). Total RNA was reverse transcribed using the Multiscribe reverse transcriptase (Applied Biosystems, Courtaboeuf, France), according to the manufacturer's protocol. PCR primers for the selected genes and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control were obtained from published references or designed using the PRIMER3 software (Table I) (24-26). Real-Time PCR was run in a LightCycler Instrument (Roche) using the QuantiTect® SYBRt® Green PCR Kit (Qiagen, GmbH, Germany) following previously described protocols (27). For each target gene, mRNA levels were normalized using the reference gene GAPDH.

Statistical analysis

Spearman's rank correlation analysis was used to test the relationship between age and differentiation and the differences among the follow-up times. The Mann Whitney test was performed to assess differences between means of the 2 types. General linear model for repeated measures with Sidak correction for multiple comparisons and type and age grouping as factors was performed to test differences of the scores at different follow-up times and the influence of age and type. For all tests $p<0.05$ was considered significant. Statistical Analysis was carried out by the Statistical Package for the Social Sciences (SPSS) software version 15.1 (SPSS Inc., Chicago, USA).

RESULTS

Flow cytometric analysis

BMCs and freshly isolated MNCs analyzed by FACS before seeding was positive for CD90, CD105, CD106, CD146 (Fig. 1A). The expression of these markers is similar between the two cell population and is not related to age (data not shown).

Colony-Forming Units-Fibroblast (CFU-F) assay

The functional capacity of BMCs and MNCs was determined by measuring the colony-forming activity. The CFU-F test showed that the mean number of colonies obtained with the two different cell populations was similar (Fig. 1B). No differences were observed among cultures of patients of different ages (data not shown).

Alcian Blue staining

During chondrogenic differentiation, an extracellular matrix deposition was evident on day 28 in both BMC and MNC cells (Fig. 2A). A slight difference was observed among patients of different ages (data not shown).

Alizarin Red staining

During osteogenic differentiation the calcium deposition was evident starting from day 21 and particularly on day 28 in both BMC and MNC cells. An evident difference was observed among patients

Table I. *List of primers used in Real-Time RT-PCR.*

Gene	Primer sequences (5'-3')	Amplicon size (base pairs)	Annealing temperature (°C)	References[a]
Aggrecan	5'-TCG AGG ACA GCG AGG CC 3'-TCG AGG GTG TAG CGT GTA GAG A	85	60	47
Type II collagen	5'-GAC AAT CTG GCT CCC AAC 3'-ACA GTC TTG CCC CAC TTA C	257	60	PRIMER 3
Sox-9	5'-GAG CAG ACG CAC ATC TC 3'-CCT GGG ATT GCC CCG A	118	60	PRIMER 3
ALP	5' GGA AGA CAC TCT GAC CGT 3' GCC CAT TGC CAT ACA GGA	152	60	PRIMER 3
BSP	5' CAGTAGTGACTCATCCGAAG 3'CATAGCCCAGTGTTGTAGCA	158	60	PRIMER 3
OC	5' GCA GCG AGG TAG TGA AGA 3' TCC TGA AAG CCG ATG TGG	148	60	PRIMER 3
GAPDH	5'-TGG TAT CGT GGA AGG ACT CAT GAC 3'-ATG CCA GTG AGC TTC CCG TTC AGC	190	60	46

[a]*Primer sequences were obtained from published references where indicated or designed using PRIMER 3.*

of different ages in a high percentage of cases evaluated (Fig. 2B).

Analysis of mRNAs expression by Real-Time PCR: Chondrogenesis

To investigate the chondrogenic differentiation potential of both BMCs and MNCs, type II collagen, Sox-9 and aggrecan mRNA were analyzed by quantitative RT-PCR on day 0 and 28, since in the authors' experience before this time mRNA chondrogenic-specific markers are very poorly expressed. No significant differences were observed between the two cell groups analyzed at all the experimental times evaluated. Collagen type II mRNA, which was almost undetectable on day 0 in both BMCs and MNCs, showed a very light increase on day 28 in the first group while a more evident, even not significant, expression on the same day in the second one (Fig. 3A). Sox-9, a transcriptional factor of collagen type II, was significantly highly expressed on day 28 compared to day 0 ($p<0.0005$) (Fig. 3B). Aggrecan mRNA was highly expressed on day 28 compared to day 0 with significant values ($p<0.0005$) (Fig. 3C). For these chondrogenic markers no differences were observed among patients of different ages (data not shown).

Analysis of mRNAs expression by Real-Time PCR:Osteogenesis

No difference between BMC and MNC groups was observed for ALP, BSP and OC mRNA expression at all the experimental times evaluated. In general, ALP was significantly higher on day 21 and day 28 compared to day 0 ($p<0.0005$) although it tended to decrease from day 21 until day 28 (Fig. 4A). These differences were influenced by age since the higher increase was evident for younger patients (<25 years) compared to "older ones" (>25 years) as was the decrease which was more evident for the first group ($p=0.037$) (Fig. 4B). BSP increased significantly on day 21 ($p<0.0005$) and 28 ($p<0.013$) compared to day 0 in both cell populations (Fig. 4C). Moreover, inside each group, a significant difference between younger patient and older ones was observed ($p<0.035$) since in the former BSP increased at high levels up to 28 days (Fig. 4D). Osteocalcin mRNA

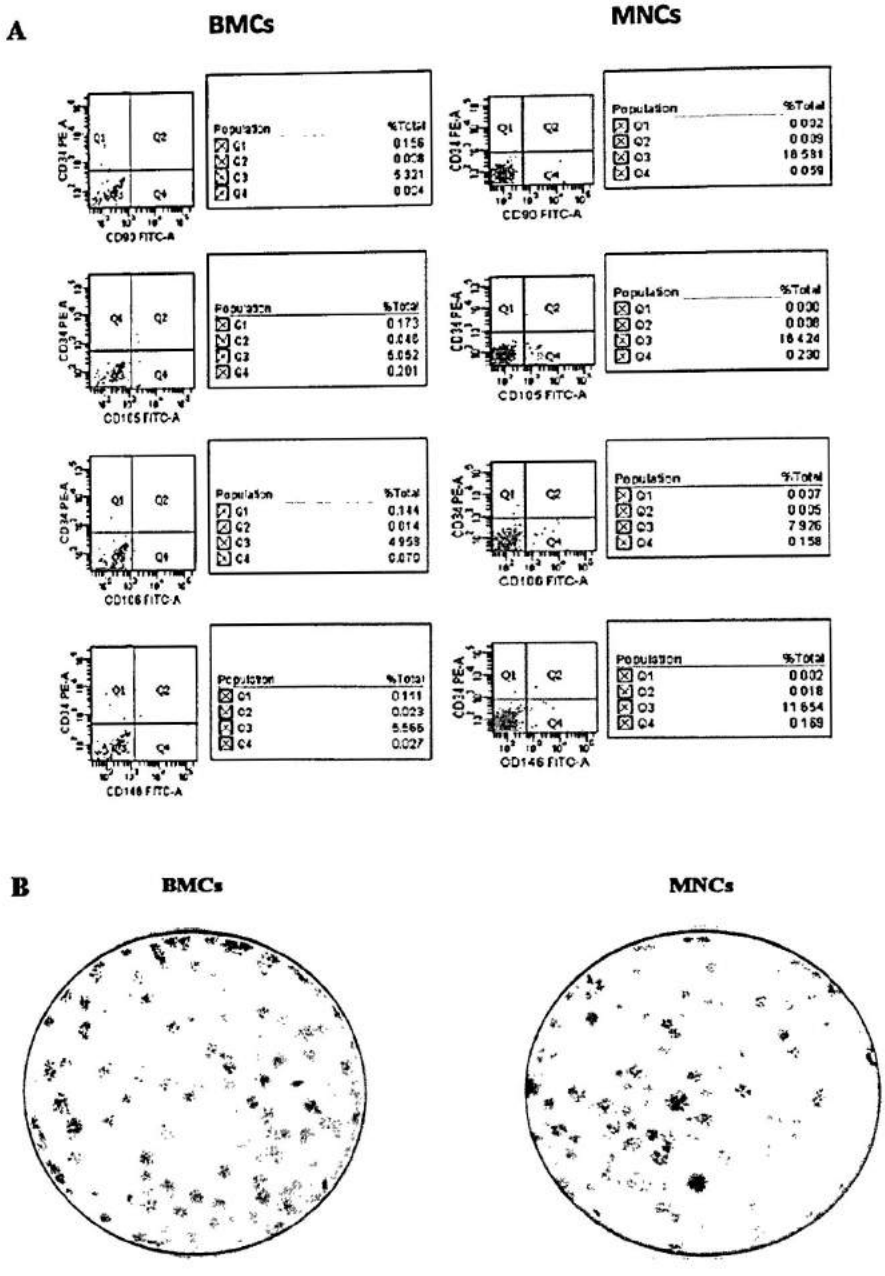

Fig. 1. *A) Flow cytometric analyses of CD90+, CD105+, CD106+ and CD146+ evaluated on CD34 and CD45 negative cells. B) Colonies forming Units. 14 days after initial seeding of 5x10⁵ BMCs and MNCs; no differences between the two populations analyzed are present. Two representative colonies in CFU-F assay are shown.*

was slightly highly expressed on day 21 compared to day 0 and significantly more highly expressed on day 28 compared to day 0 ($p<0.049$) (Fig. 4E). No significant differences were observed among patients of different ages (data not shown).

DISCUSSION

Currently, there is no reliable reconstructive technique to repair osteochondral articular defects anatomically (1). Cartilage and bone repair requires a

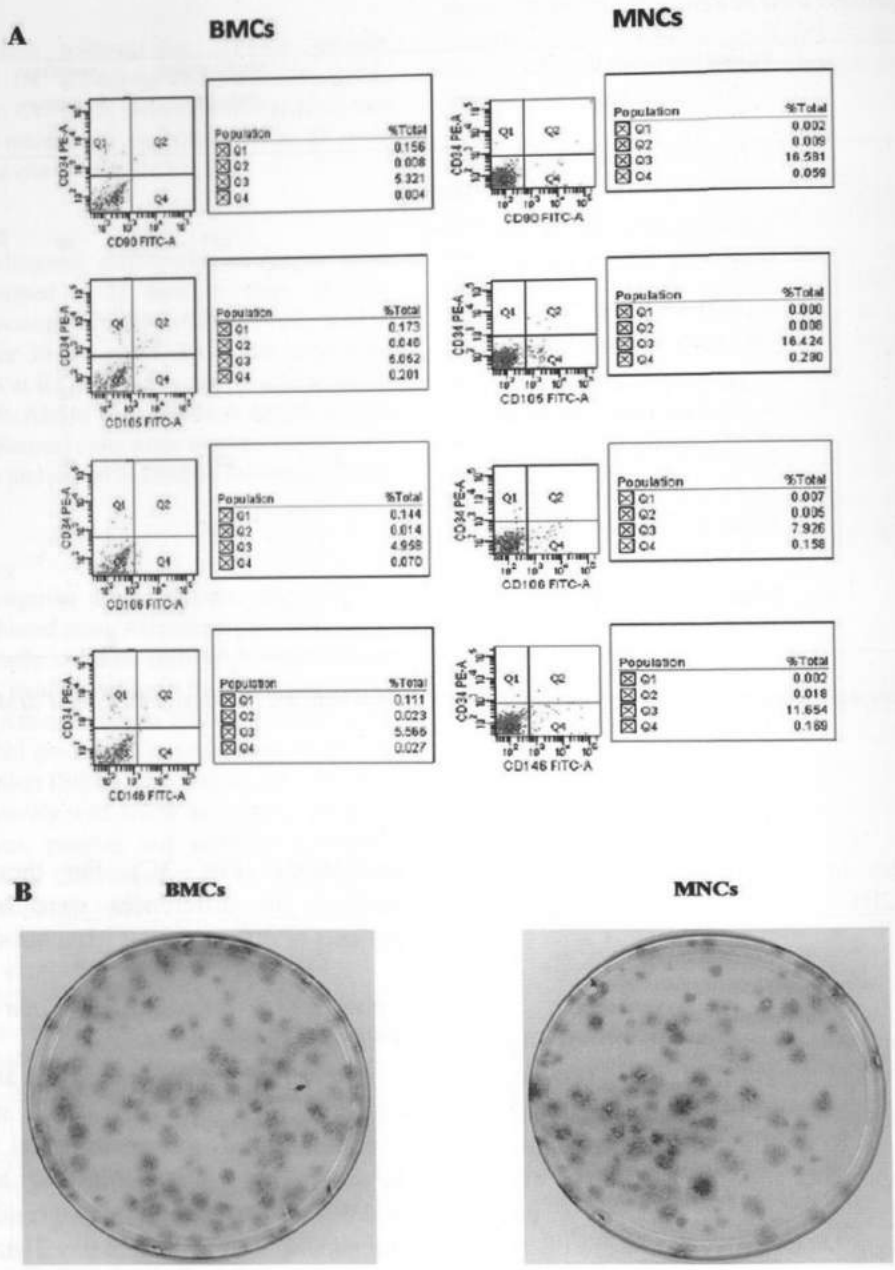

Fig. 1. *A) Flow cytometric analyses of CD90+, CD105+, CD106+ and CD146+ evaluated on CD34 and CD45 negative cells. B) Colonies forming Units. 14 days after initial seeding of $5x10^5$ BMCs and MNCs; no differences between the two populations analyzed are present. Two representative colonies in CFU-F assay are shown.*

was slightly highly expressed on day 21 compared to day 0 and significantly more highly expressed on day 28 compared to day 0 ($p<0.049$) (Fig. 4E). No significant differences were observed among patients of different ages (data not shown).

DISCUSSION

Currently, there is no reliable reconstructive technique to repair osteochondral articular defects anatomically (1). Cartilage and bone repair requires a

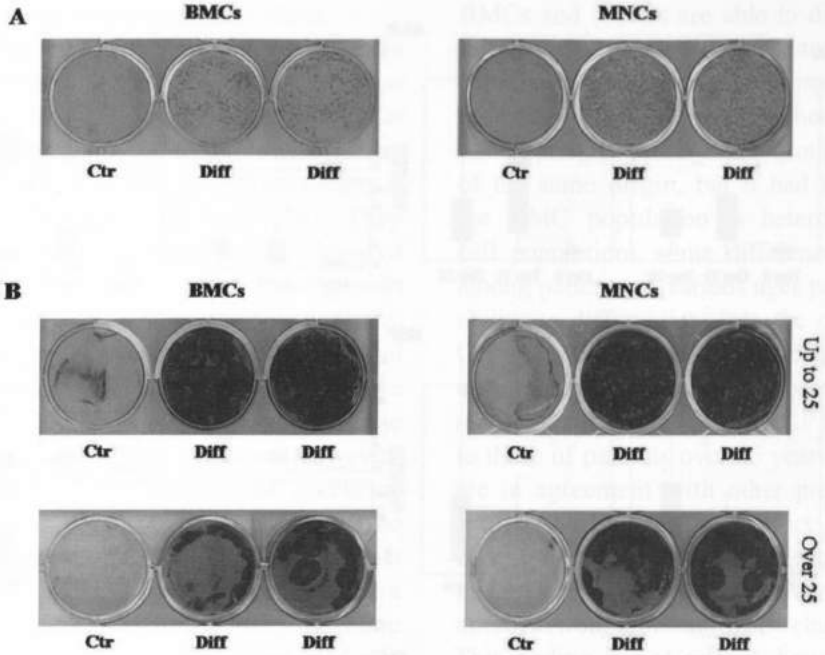

Fig. 2. *A) Alcian Blue staining of BMCs and MNCs cultured in the absence (Ctr) or presence of TGF-β1 (Diff) at 28 days. Diff samples are in duplicate. No differences between BMCs and MNCs are present.* ***B)*** *Alizarin Red S staining of BMCs and MNCs of representative patients cultured in the absence (Ctr) or presence of ascorbate-2 phosphate, β-glycerophosphate and dexamethasone (Diff) at 28 days. Diff samples are in duplicate. In the upper panel cells from a patient <25 years were seeded, whereas in the lower panel, cells from a patient >25 years were seeded. There is a visible different staining between the cells from the patients of different ages.*

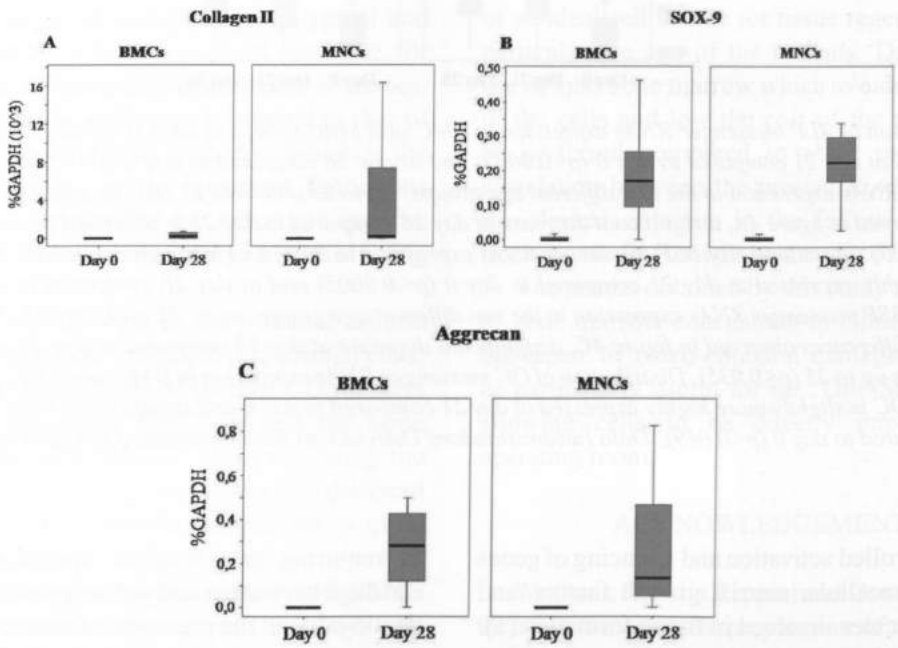

Fig. 3. *Distribution of Type II collagen (A), Sox-9 (B) and Aggrecan (C) messenger RNAs expression in BMCs and MNCs at day 0 and day 28. Sox-9 is more highly expressed at day 28 compared to day 0 ($p<0.0005$) such as Aggrecan ($p<0.0005$). Data (mean±standard deviation) were normalized to GAPDH.*

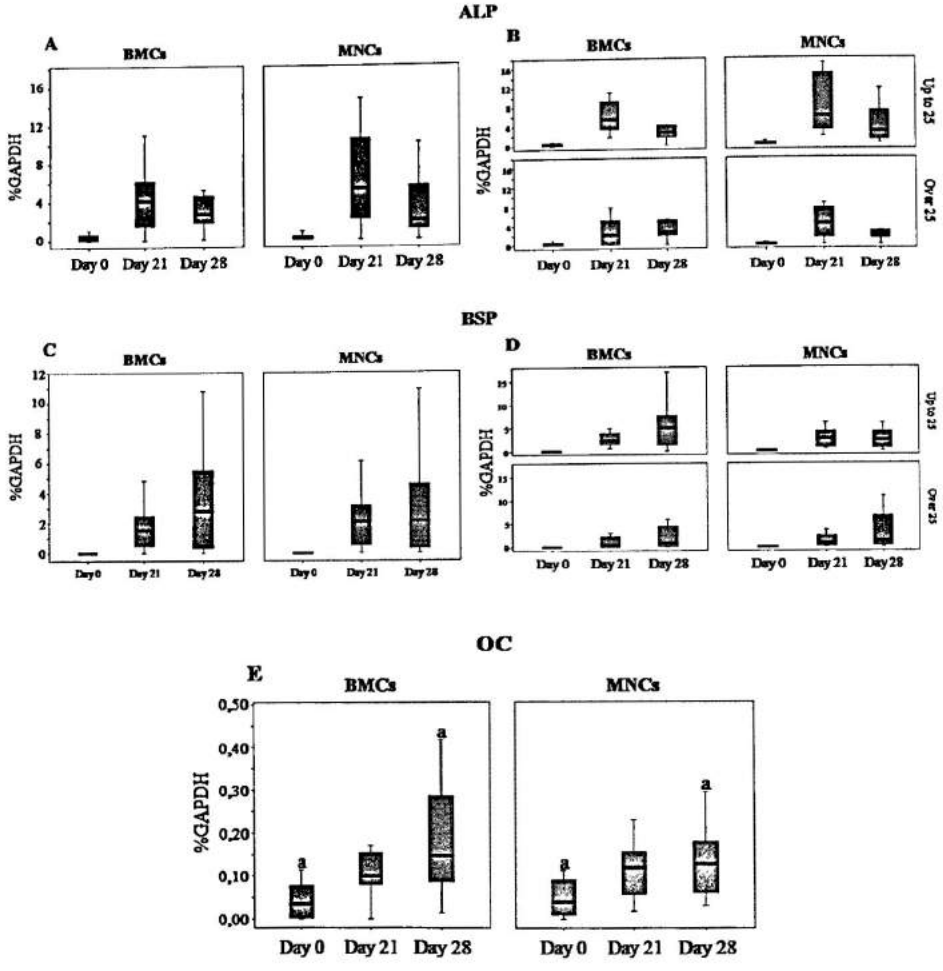

Fig. 4. *Distribution of ALP messenger RNAs expression in BMC and MNC groups at days 0, 21 and 28 (A); ALP is more highly expressed at day 21 compared to day 0 ($p<0.0005$) and at day 28 compared to day 0 ($p<0.0005$). Distribution of ALP messenger RNAs expression in the two different age groups: up to 25 and over 25 (**B**); in addition to the significant differences observed in figure 4A, a significant decrease at day 28 compared to day 21 is observed in the group of patient up to 25 ($p<0.037$). Distribution of BSP messenger RNAs expression in BMC and MNC groups at day 0, 21 and 28 (**C**); BSP is more highly expressed at day 21 compared to day 0 ($p<0.0005$) and at day 28 compared to day 0 ($p<0.013$). Distribution of BSP messenger RNAs expression in the two different age groups: up to 25 and over 25 (**D**); in addition to the significant differences observed in figure 4C, a significant decrease at day 28 compared to day 21 is observed in the group of patients up to 25 ($p<0.035$). Distribution of OC messenger RNAs expression in BMC and MNC groups at days 0, 21 and 28 (**E**). OC is slightly more highly expressed at day 21 compared to day 0 and significantly more highly expressed at day 28 compared to day 0 ($p<0.049$). Data (mean±standard Ddeviation) were normalized to GAPDH.*

multistep controlled activation and silencing of genes producing extracellular matrix, growth factors, and adhesion molecules involved in tissue formation (1). The use of autologous chondrocyte transplantation has produced successful results in recent years (28). However, this technique has some limitations, such as requiring two surgical operations for healthy cartilage harvesting and cell implantation and it is not employable in the presence of osteochondral defects which require the regeneration of both cartilage and bone tissues (1). This has led to the search for other cell types that can be obtained from the patient

without performing an arthroscopy procedure.

MSCs are a fascinating source for regenerative medicine because they can be harvested in a less invasive manner, easily isolated and expanded *in vitro*. MSCs have proven to be able to differentiate, after appropriate induction, into several mesodermal tissues including cartilage and bone (29). They provide tissue protection and repair by the release of paracrine molecules including cytokines and growth factors (30). Nevertheless, in bone marrow aspirate, MSCs account for a very small fraction of the total population of nucleated cells; therefore, innovative in-depth research has paved the way for the clinical use of concentrate bone marrow · However, despite the increase in the number of precursor cells obtained by the concentration procedure, the rationale for using total bone marrow resides in its peculiar characteristics that identify it as an orchestra of cells and signals. As reported above, the bone marrow niche which comprises HSCs and MSCs is responsible for the regeneration of tissues. However, it is certain that the composition of cell types and extracellular matrix components in the bone marrow varies extensively over a lifetime. These changes are reflected also by decreased bone formation .

The present study was aimed at characterizing human BMCs, by analyzing their phenotypical and functional features to further support their use for the regeneration of damaged osteochondral tissues. The behavior of these cells was compared to that of MNCs isolated by the Ficoll procedure which is the gold standard but has some important limitations such as the time needed for the procedure and the requirement of Good Laboratory Manufacturing practice (GMP).

Taking into account one of the minimal criteria for defining multi-potent mesenchymal stromal cells, that refers to the expression of specific CD markers (31), our data show that BMCs present the same CD pattern as isolated MNCs. However, being the MSC population extremely low in the two different cell populations a quantitative comparison is quite impossible.

Concerning the clonogenic capacity of BMCs and MNCs, our results demonstrated that both cell populations are able to form CFU-F colonies with a similar ability.

Concerning the differentiation potential, both BMCs and MNCs are able to differentiate *in vitro* towards chondrogenic and osteogenic lineages as testified by the expression of specific extracellular molecules during culture. These results are not be surprising since both groups comprise cells of the same origin, but it had to be shown since the BMC population is heterogeneous. In both cell populations some differences are highlighted among patients of various ages particularly for their ability to differentiate into the osteogenic lineage. Cells from patients up to 25 years old express, during their differentiation process, higher levels of osteogenic markers such as ALP and BSP compared to those of patients over 25 years old. These results are in agreement with other previous data giving further evidence of the effect of donor age on differentiation, particularly towards the osteogenic lineage (13), and confirm the combination of niche microenvironment intrinsic changes and aging. This finding is not related, however, to a specific mesenchymal phenotype since no correlations were observed between the expression of cellular surface markers and that of specific molecules involved in the differentiation processes. Based on the results of this and other similar studies, it is evident that many variables must be taken into account for the choice of an ideal cell source for tissue regeneration and in particular the age of the patients. Despite this, the use of total bone marrow which avoids the expansion of the cells and low the cost of the procedure may be preferred compared to other procedures. The correlation between the present *in vitro* results and the clinical outcome in the follow-up of patients treated with BMCs will be the aim of future studies.

The results obtained by this study support the use of bone marrow concentrate in clinical practice for the repair of osteo-chondral damage, which might be particularly useful for the "One-Step" procedure allowing cells to be directly implanted in the operating room.

ACKNOWLEDGEMENTS

We thank Patrizia Rappini and Graziella Salmi for their assistance in the preparation of the manuscript and Luciano Pizzi for his technical assistance. We also thank Dr Elettra Pignotti for statistical analysis and Dr Keith Smith for the revision of English

language. This work was partially supported by grants from Progetto Regione Emilia-Romagna Università 2007-2009 "Regenerative medicine in osteo-articular diseases".

REFERENCES

1. Buckwalter JA, Rosenberg LC, Hunziker EB. Articular cartilage: composition, structure, response to injury and methods of repair. In Articular cartilage and knee joint function: basic science and arthroscopy. JW Ewing eds Raven Press 1990; p.19.
2. Clarke HD, Scott WN. The role of debridement: through small portals. J Arthroplasty 2003; 18:10-3.
3. Takao M, Ochi M, Naito K, Uchio Y, Kono T, Oae K. Arthroscopic drilling for chondral, subchondral, and combined chondral-subchondral lesions of the talar dome. Arthroscopy 2003; 19:524-30.
4. Steadman JR, Rodkey WG, Rodrigo JJ. Microfracture: surgical technique and rehabilitation to treat chondral defects. Clin Orthop Relat Res 2001; 391:(S)362-9.
5. Ritsilä VA, Santavirta S, Alhopuro S,. Periosteal and perichondral grafting in reconstructive surgery. Clin Orthop Relat Res 1994; 302:259-65.
6. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331:889-95.
7. Kon E, Verdonk P, Condello V, Delcogliano M, Dhollander A, Filardo G, Pignotti E, Marcacci M. Matrix-assisted autologous chondrocyte transplantation for the repair of cartilage defects of the knee: systematic clinical data review and study quality analysis. Am J Sports Med 2009; 37:156-66.
8. Ronzière MC, Perrier E, Mallein-Gerin F, Freyria AM. Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. Biomed Mater Eng 2010; 20:145-58.
9. Heng BC, Cao T, Lee EH. Directing stem cell differentiation into the chondrogenic lineage in vitro. Stem Cells 2004; 22:1152-67.
10. Grigolo B, Lisignoli G, Desando G. Osteoarthritis treated with mesenchymal stem cells on hyaluronan-based scaffold in rabbit. Tissue Eng Part C Methods 2009; 15:647-58.
11. Marcacci M, Kon E, Moukhachev V, Lavroukov A, Kutepov S, Quarto R, Mastrogiacomo M, Cancedda R. Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. Tissue Eng 2007; 13:947-55.
12. Chambers SM, Goodell MA. Hematopoietic stem cell aging: wrinkles in stem cell potential. Stem Cell Rev 2007; 3:201-11.
13. Roobrouck VD, Ulloa-Montoya F, Verfaillie CM. Self-renewal and differentiation capacity of young and aged stem cells. Exp Cell Res 2008; 314:1937-44.
14. Oreffo RO, Bennett A, Carr AJ, Triffitt JT. Patients with primary osteoarthritis show no change with ageing in the number of osteogenic precursors, Scand J Rheumatol 1998; 27:415-24.
15. Quarto R, Thomas D, Liang CT. Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif Tissue Int 1995; 56:123-9.
16. Indrawattana N, Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, Tanaka J, Bunyaratvej A. Growth factor combination for chondrogenic induction from human mesenchymal stem cell, Biochem Biophys Res Commun 2004; 320:914-9.
17. Chimal-Monroy J, Rodriguez-Leon J, Montero JA, Gañan Y, Macias D, Merino R. Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. Dev Biol 2003; 257:292-301.
18. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 2005; 105:2631-9.
19. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 1978; 4:7-25.
20. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature 2001; 414:98-104.
21. Velazquez OC. Angiogenesis and vasculogenesis: inducing the growth of new blood vessels and wound healing by stimulation of bone marrow-derived progenitor cell mobilization and homing. J Vasc Surg 2007; 45:39-47.
22. Tonti GA, Mannello F. From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera. Int J Dev Biol 2008; 52:1023-32.

23. Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet culture system. Exp Hematol 2004; 32:502-9.
24. Blanco FJ, Geng Y, Lotz M. Differentiation-dependent effects of IL-1 and TGF-β on human articular chondrocyte proliferation are related to inducible nitric oxide synthase expression. J Immunol 1995; 154:4018-26.
25. Martin I, Jakob M, Schäfer D, Dick W, Spagnoli G, Heberer M. Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Ostearthritis Cart 2001; 9:112-8.
26. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana 2000; p.365.
27. Cavallo C, Desando G, Columbaro M. Ferrari A, Zini N, Facchini A, Grigolo B. Chondrogenic differentiation of bone marrow concentrate grown onto a hylauronan scaffold: rationale for its use in the treatment of cartilage lesions. J Biomed Mat Res Part A 2012; doi10:1002.
28. Harris JD, Siston RA, Pan X, Flanigan DC. Autologous chondrocyte implantation: a systematic review. J Bone Joint Surg Am 2010; 92:2220-33.
29. Caplan AI. Mesenchymal stem cells. J Orthop Res 1991; 9:641-50.
30. Schutze N, Noth U, Schneidereit J, Hendrich C, Jakob F. Differential expression of CCN-family members in primary human bone marrow-derived mesenchymal stem cells during osteogenic, chondrogenic and adipogenic differentiation. Cell Commun Signal 2005; 31-12.
31. Dominici M, Le Blanc K, Mueller I. Minimal criteria for defining multi-potent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8:315-7.