Cytotherapy

GMP-grade preparation of biomimetic scaffolds with osteo-differentiated autologous mesenchymal stromal cells for the treatment of alveolar bone resorption in periodontal disease

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Background

Periodontal disease is a degenerative illness that leads to resorption of the alveolar bone. Mesenchymal stromal cells (MSC) represent a novel tool for the production of biologic constructs for the treatment of degenerative bone diseases. The preparation of MSC differentiated into osteogenic lineage for clinical use requires the fulfillment of strict good manufacturing practice (GMP) procedures.

Methods

MSC were isolated from BM samples and then cultured under GMP conditions. MSC were characterized phenotypically and for their differentiative potential. Cells were seeded onto collagen scaffolds (Gingistat) and induced to differentiate into osteogenic lineages using clinical grade drugs compared with standard osteogenic supplements. Alizarin Red S stain was used to test the deposition of the mineral matrix. Standard microbiologic analysis was performed to verify the product sterility.

Results

The resulting MSC were negative for CD33, CD34 and HLA-DR but showed high expression of CD90, CD105 and HLA-ABC (average expressions of 94.3%, 75.8% and 94.2%, respectively). Chondrogenic, osteogenic and adipogenic differentiation potential was demonstrated. The MSC retained their ability to differentiate into osteogenic lineage when seeded onto collagen scaffolds after exposure to a clinical grade medium. Cell numbers and cell viability were adequate for clinical use, and microbiologic assays demonstrated the absence of any contamination.

Discussion

In the specific context of a degenerative bone disease with limited involvement of skeletal tissue, the combined use of MSC, exposed to an osteogenic clinical grade medium, and biomimetic biodegradable scaffolds offers the possibility of producing adequate numbers of biologic tissue-engineered cell-based constructs for use in clinical trials.

Keywords

GMP, MSC, periodontal disease, tissue engineering.

Introduction

Periodontal disease is an inflammatory condition with multifactorial etiology that has a high incidence in the population, depending on age, external behavioral habits such as food and smoking, and the concomitant presence of other chronic systemic diseases. The disease is chronic and degenerative and leads to the destruction of the periodontal apparatus, with resorption of the alveolar bone,

periodontal ligament, cementum and gingiva. Eventually, the disease leads to the loss of teeth, with severe consequences for the stomatognatic apparatus [1,2].

Treatment of severe forms of the disease involves the restoration of lost supporting tissues, including new alveolar bone, new cementum and a new periodontal ligament. However, at the present time, therapeutic approaches for regenerating the periodontal tissues, such as guided tissue regeneration (GTR), guided bone regeneration (GBR), enamel matrix derivative (EMD) and the use of various growth factors [3–5], are not satisfactory, and different researchers have focused their attention on new techniques of tissue engineering, such as the manufacture of synthetic materials and cell therapy [6,7].

Within the BM stroma there is a subset of non-hematopoietc cells referred as mesenchymal stem cells or mesenchymal stromal cells (MSC) [8]. MSC are defined as undifferentiated cells able to self-renew with a high proliferative capacity. They can differentiate *in vitro* and *in vivo* into various mesoderm-type cell lineages [9–11] and into non-mesodermal lineages [12,13].

MSC have generated a great deal of interest because of their potential use in regenerative medicine, in particular for the treatment of degenerative diseases of the skeletal apparatus. The multipotent capacity of these cells, the possibility of being easily isolated in BM aspirates, their high *ex vivo* expansion potential [14,15] and their immunomodulatory properties [16] place these cells among the best candidates for cell therapy for regenerating injured skeletal tissues. Tissue engineering for correction of bone defects by autologous MSC transplantation is one of the most promising concepts being developed in regenerative medicine to treat degenerative and age-related diseases and traumas. In fact, this medical strategy eliminates problems linked with the morbidity observed after implants of autologous bone grafts and the immunogenicity of allogenic grafts [17].

One of the most attractive strategies of tissue engineering involves the use of three-dimensional scaffolds to support the growth and differentiation of MSC, with the aim of promoting tissue regeneration when implanted into the affected areas. These constructs have been used in pre-clinical models, showing promising results for bone even though carried out on different animal species, different types of bone defects and different type of scaffolds, offering various examples of therapeutic value in bone regeneration, and showing good cellular grafting and direct involvement of the inoculated MSC in the process of new tissue formation [18–21].

Functional bone healing and the speed of recovery seem to improve if bone defects are treated with *ex vivo*-expanded MSC placed on three-dimensional matrices and afterwards implanted into the bone defects [22–25], instead of using the unseparated total BM cell product [26,27]. Moreover, further improvement can be observed when MSC are expanded *ex vivo* and induced to differentiate into the osteogenic lineage after exposure to osteogenic medium, enriched with dexamethasone alone or dexamethasone, ascorbic acid and sodium glycerol-phosphate [28–30].

The implant of scaffolds with MSC has given positive results in humans, even if few clinical trials have been performed [31]. In a limited number of patients, Quarto *et al.* [32] showed a complete consolidation between the implant and the host bone, occurring between 5 and 6 months after surgery. Ohgushi *et al.* [33] carried out a preliminary study of tissue-engineered prostheses on three patients suffering from ankle arthritis and followed their progress for 2 years. They employed *ex vivo*-expanded MSC to a ceramic ankle prostheses and induced them into osteogenic differentiation *in vitro* for 2 weeks before inoculating them into the affected areas. They finally demonstrated a stable interface between the ceramic surface and the host bone. In these clinical trials, no relevant immunity reaction has been described or any infection at the site of implant or any systemic side-effects.

The aim of the present study was to validate, under good manufacturing practice (GMP) conditions, a protocol of tissue engineering applied to bone alveolar regeneration for periodontal disease, inducing osteogenic differentiation of MSC on a biomimetic clinical grade scaffold (Gingistat) by using a combination of GMP-grade drugs. The study shows that BM-autologous *ex vivo*-expanded MSC can be induced to differentiate into osteogenic cells when stimulated with clinical grade drugs and placed on biomimetic scaffolds. Sufficient cell numbers are obtained for implant into bone defects of various sizes. Certification of the quality of a clinical grade cell product needs analysis of MSC identity, assurance of the absence of any micro-biologic contamination and reliable and reproducible assays to measure the grade of osteogenic differentiation.

Methods

Isolation and culture of MSC

BM cells were harvested from the iliac crest of four healthy donors who each underwent BM collection for a

related patient. For our experiments, we used unfiltered BM collection bags. All cell expansions were performed in a GMP facility (Cell Therapy Laboratory, 'Stefano Verri', Monza, Italy).

Collection bags were washed with PBS (LiStarFish, Milan, Italy) several times to harvest the maximum amount of BM. Density-gradient (Ficoll-Hypaque, GEheathcare, Milan, Italy) separation was performed to isolate mononuclear cells. Mononuclear cells were resuspended in a GMP-grade culture medium, composed of low-glucose DMEM (DMEM-LG; LiStarFish) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 2 mm l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (LiStarFish) and plated in culture flasks at 1.6×10^5 cells/cm^2. The medium was changed twice a week and, as the culture reached around 80% confluence, cells were trypsinized and subsequently split and subcultured until passage 3 (p3). Cell viability was evaluated at each passage by trypan blue count. The fold increase of each culture was expressed with respect to the number of cells at passage 1 (p1) and p3 (p3/p1).

Flow cytometry analysis of MSC

Expanded cells were detached with trypsin (LiStarFish), washed with PBS and resuspended at a final concentration of 1×10^6 cells/100 μL in PBS. Cells were incubated for 25 min in the dark, at room temperature, with 5 μL of each MAb: PE-conjugated anti-CD33 MAb (Chemicon, Temecula, CA, USA), PE-conjugated anti-CD34 MAb (Chemicon), FITC-conjugated anti HLA-DR MAb (Chemicon), FITC-conjugated anti HLA-ABC MAb (Dako, Glostrup, Denmark), PE-conjugated anti-CD90 MAb (Chemicon) and FITC-conjugated anti-CD105 MAb (Chemicon). Labeled cells were washed with PBS, resuspended in PBS and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Josè, CA, USA). At least 10 000 events were acquired for each analysis, after gating on viable cells, and isotype-control Ab were used to set the appropriate gates.

Analysis of MSC differentiation into mesengenic lineages

Osteogenic differentiation

Cells were seeded at 4×10^3 cells/cm^2 on cover glasses in culture medium. After reaching a subconfluence level, cells were incubated in culture medium alone or standard osteogenic medium (OS) consisting of culture medium with the addition of osteogenic supplements, i.e. 100 nM dexamethasone (Applichem GmbH, Darmstadt, Germany), 10 mM β-glycerol-phosphate (Applichem) and 0.05 mM 2-phosphate-ascorbic acid (Sigma Chemicals Co, St. Louis, MO, USA). Fresh medium was added twice a week.

After 4 weeks of culture, osteogenic differentiation was assessed by immunofluorescence. Selected cultures were fixed for 10 min with 4% paraformaldehyde (PFA; Sigma) and treated for 10 min with 0.1 m glycine (Sigma) in PBS, followed by a 1 h incubation at room temperature with a blocking solution (5% BSA, 0.5% Triton X-100 in PBS) and subsequently a 30-min incubation with 1 mg/mL RNAse (Sigma) in blocking solution.

Cover glasses were then incubated overnight at 4°C with primary anti-osteopontin Ab (Chemicon) at a 1:200 dilution, washed and incubated for 1 h at room temperature with the appropriate FITC-conjugated secondary Ab (1:100 dilution) (Rockland, Gilbertsville, PA, USA) and 2.5 μg/mL propidium iodide (PI). After a final washing, cover glasses were mounted with glycerol. Microscopy analysis was performed with laser confocal microscopy (Radiance 2100; BioRad Laboratories, Hercules, CA, USA).

Chondrogenic differentiation

Adherent cells were trypsinized and subsequently counted. Aliquots of 2.5×10^5 cells were resuspended in chondrogenic differentiation medium, centrifuged in 15-mL polypropylene conical tubes and grown as a pelletted micromass at 37°C, 5% CO_2.

Chondrogenic differentiation medium consisted of high-glucose DMEM (DMEM-HG; (BioWhittaker, Bergamo, Italy) supplemented with ITS + premix (Collaborative Biomedical Products, Bedford, MA, USA; insulin, transferrin, selenous acid at 6.25 mg/mL, linoleic acid at 5.35 mg/mL and BSA at 1.25 mg/mL), pyruvate (1 mM; Sigma), 2-phosphate-ascorbic acid (50 μg/mL), 100 nM dexamethasone and 10 ng/mL TGFβ$_3$ (10 ng/mL; Peprotech, Rocky Hill, NJ, USA). Media were changed twice a week. After 6 weeks, the spheroid cell masses were fixed in PFA for 30 min and embedded in paraffin. Afterwards, 7-μm sections were cut. Sections were stained with hematoxylin-eosin (H&E; Sigma) for the evaluation of cell morphology in pellets, and sulfated glycosaminoglycans (GAG) were visualized by staining with Safranin O 0.1% (Sigma) for 5 min.

Adipogenic differentiation

Expanded cells were seeded into dishes at a density of 5×10^3 cells/cm^2 in culture medium. The day after, the medium was switched to adipogenic induction medium (AIM), consisting of DMEM-HG supplemented with 10% FBS, 1 µM dexamethasone, 100 µM indomethacin (Sigma), 500 µM 3-isobutyl-1-methylxantine (IBMX) (Sigma) and 10 µg/mL insulin (Sigma), while control cultures were concomitantly performed in DMEM-HG 10% FBS. On day 10, AIM was replaced with adipogenic maintenance medium (AMM), which consisted of DMEM-HG 10% FBS supplemented with 10 µg/mL insulin. Media were changed twice a week. After 1 week, adipogenic cultures were washed with PBS and fixed in PFA 4% for 10 min before staining with Oil Red O (Sigma), a common fat deposit stain.

GMP preparation of clinical grade scaffolds with differentiated MSC

In order to validate a GMP-grade production protocol for clinical application, primary cultures of BM mononuclear cells were established under GMP conditions. Expanded cells were released from culture dishes at p3 and used either for osteogenic differentiation onto collagen scaffolds or culture dishes, in order to verify the beginning of the mineralization process. Clinical grade osteogenic medium (DRUGS) consisted of DMEM-LG 10% FBS supplemented with 100 nM dexamethasone, 0.05 mM ascorbic acid and 10 mM sodium glycerol-phosphate (all provided by FARVE, Altavilla, Vicentina, Italy), produced according to European Pharmacopoeia indications. Clinical grade osteogenic medium was compared with the OS medium previously described.

Seeding of MSC onto collagen scaffolds

We used the biomimetic clinical grade scaffold Gingistat® (Vebas, Milan, Italy), a sponge made of lyophilized (type I) collagen, whose structure and degradation time have already been described elsewhere [34]. Collagen sponges were cut, under sterile conditions, into $5 \times 5 \times 5$-mm cubes. MSC were then resuspended in the culture medium at a concentration of 5×10^6 cells/mL and 10^6 cells were poured onto each 125-mm^3 scaffold through a 25-gauge needle. After a 4-h incubation at 37°C with 5% CO$_2$, new medium was added. Three days later, cells were treated either with OS, DRUGS or culture medium alone (CTRL) as a negative control. Media were changed every 2 days.

Histologic analyzes of the scaffolds were performed at 14, 21, 28 and 35 days of culture. Collagen sponges were washed with PBS, fixed with 4% PFA for 45 min at room temperature, embedded in OCT (cryo-embedding matrix), frozen, and cut into 10-µm sections with cryostate. Sections were stained with H&E for the evaluation of cell integrity and mineralized matrix was visualized by staining for 30 seconds with a solution containing 1% Alizarin Red (Applichem) and 1% ammonium hydroxide (Alizarin Red S). Cells were rinsed twice with distilled water and allowed to dry completely. Excess dye was shaken off and stained sections were fixed with acetone, acetone-xylene, cleared with xylene and mounted in permanent medium.

Osteogenic differentiation of MSC on culture dishes

Cells were seeded at 4×10^3 cells/cm^2 on culture dishes in culture medium. Three days after, cells were treated with the same medium (OS, DRUGS and CTRL) utilized to treat the scaffolds. Medium was changed every 2 days. Alizarin Red S stain was performed at 14, 21, 28, 35 and 42 days of culture. PFA-fixed cells were incubated for 30 min at room temperature with Alizarin Red S. Cells were rinsed twice with distilled water and allowed to dry completely.

Microbiologic quality control of the cell product

Standard microbiologic analyses (tests for endotoxin, mycoplasma, aerobic bacteria, anaerobic bacteria and fungi) were performed before placing MSC on collagen scaffolds, according to European Pharmacopeia standards. The same pattern of tests was repeated on the scaffold medium at the end of the process of osteogenic differentiation.

Results
MSC: culture and phenotypic characterization

The first step towards validating our process of cell production was to demonstrate a reproducible and GMP-grade method of expanding a sufficient number of characterized MSC to be subsequently used on the biomimetic scaffolds. Cells were isolated from the BM of four different donors and primary cultures were established under GMP conditions. The BM cells generated a confluent layer of cells with an elongated fibroblastic shape (Figure 1a). No macroscopic differences in morphology were observed between each passage or among the different tested samples. The growth rate of each cell expansion was evaluated by trypan blue count. The
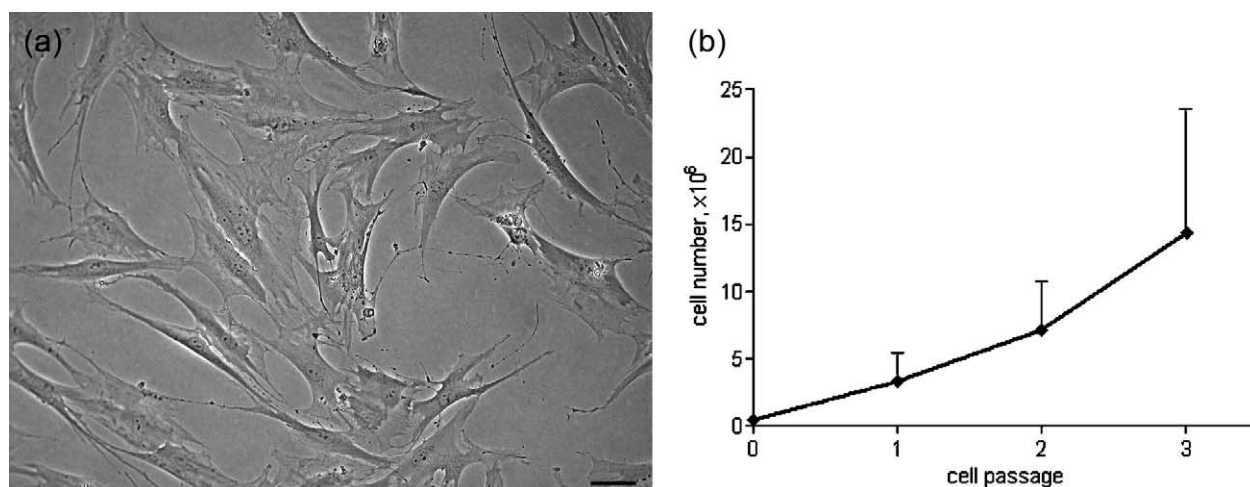
Figure 1. Culture of MSC under GMP conditions. (a) Cells at p3 show the typical fibroblastic morphology. Bar = 50 μm. (b) The growth rate of the four expansions was evaluated (average cell numbers plus SD are shown).

analysis showed high levels of viability (between 95 and 100%) in all samples and all passages analyzed. Figure 1b shows the growth rate of the different cell cultures. The results demonstrated that MSC can be expanded in sufficient numbers under GMP conditions using certified reagents. A variable expansion growth rate was observed, as expected [35–38], for the different donors tested, but it did not impair the process of production.

The second step was represented by the identification of a panel of molecular markers that could confidently identify the nature of the expanded MSC. For such a purpose, MSC were characterized by FACS analysis at p3 (Figure 2) for the expression of selected mesenchymal Ag (CD90, CD105 and HLA-ABC) and the lack of typical hematopoietic markers (CD33, CD34 and HLA-DR). At p3, MSC were constantly negative for CD33, CD34 and HLA-DR, with an Ag expression less than 2% (an average of 0.15%, 0.34% and 0.35%, respectively). On the contrary the MSC showed a high expression of CD90 (average 94.3%, range 92.2–97%), CD105 (average 75.8%, range 70–81.1%) and HLA-ABC (average 94.2%, range 91.8–97%). The results showed that the process of MSC expansion always led to a cell product, which was consistently and reproducibly characterized by a specific pattern of surface marker expression, thus confirming the nature of multipotent MSC.

Multipotentiality of MSC

Even though the phenotypic characterization of MSC is a robust reading for the analysis of cell identity, nevertheless the final demonstration of the multipotential capacity of MSC can only be tested by assessing the differentiation potential of MSC towards the three different mesengenic lineages. Cells treated with specific induction media were able to differentiate *in vitro* into osteogenic, adipogenic and chondrogenic lineages (Figure 3).

With regard to osteogenic differentiation, we evaluated osteopontin expression by immunofluorescence after 28 days of cell culture in CTRL or OS medium. Observations at high magnification revealed a cytoplasmic expression of the protein in OS cultures only (Figure 3a, b). After 3 weeks of adipogenic induction, intracellular lipid droplets were stained with Oil red O. Cells induced into chondrogenic differentiation in cell masses changed in morphology, acquiring an oval shape and increasing in size. Some of the cells were arranged in clusters dipped in an abundant extracellular matrix rich of proteoglycans and glycosaminoglycans, as confirmed by Safranin O solution staining (Figure 3e, f). The results showed that, besides being characterized by a specific expression of a panel of different surface markers, expanded MSC are characterized by a multipotent capacity to differentiate into the three mesengenic lineages.

MSC: osteogenic differentiation under GMP conditions

After demonstrating the multipotent capacity of the expanded MSC, cells were seeded onto biomimetic scaffolds and exposed to the osteogenic GMP-grade differentiation medium to demonstrate that our production process was
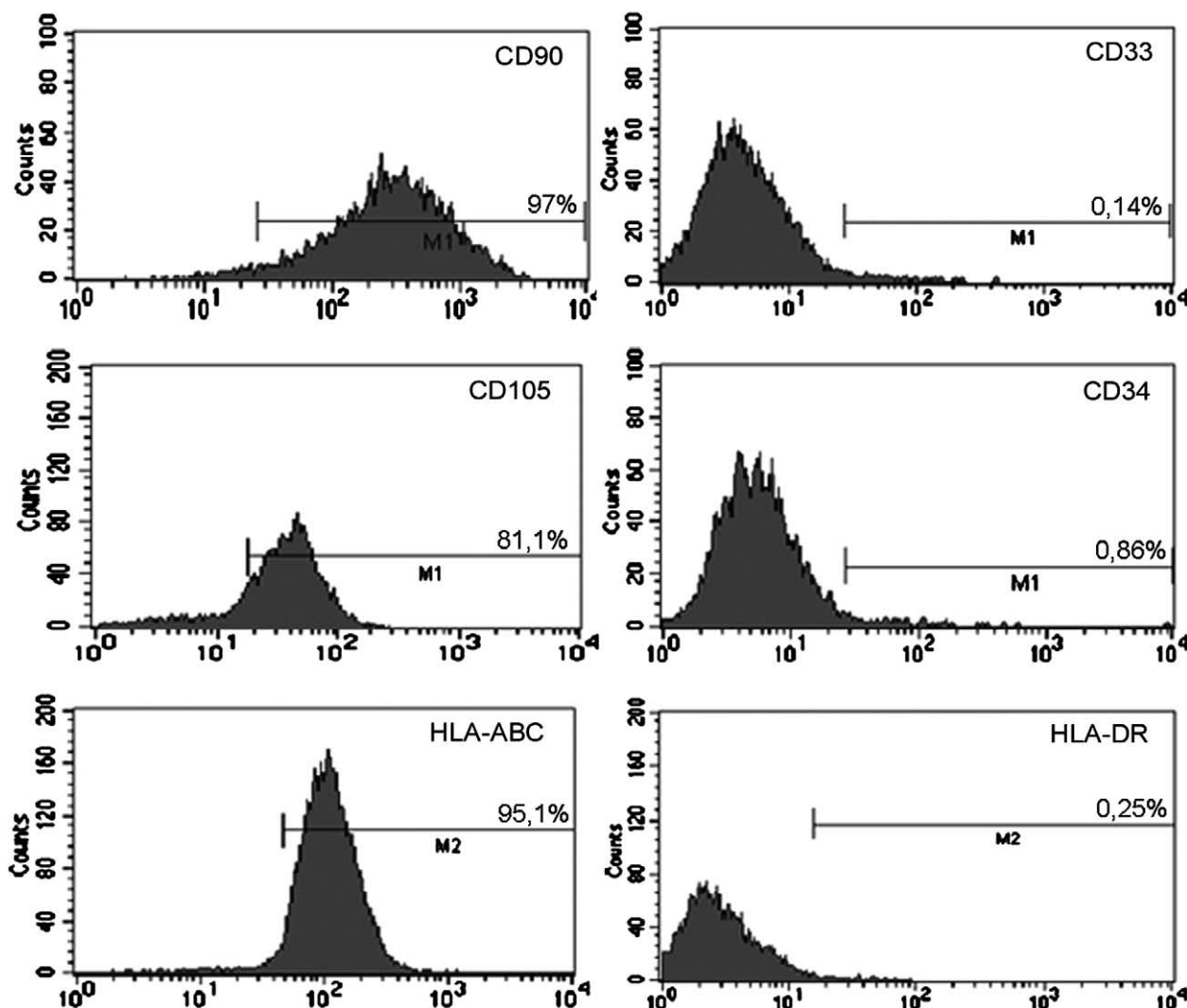
Figure 2. Immunophenotype of one representative cell expansion (out of four performed). The CD105, CD90, HLA-ABC, CD33, CD34 and HLA-DR were analyzed by FACS. The histograms show the percentages of positive cells for each single marker after gating on viable cells.

capable of generating osteo-differentiated MSC in a three-dimensional matrix. MSC at p3 were placed on culture dishes and onto collagen scaffolds. Three days later, the cells were induced to differentiate into the osteogenic lineage. DRUGS was compared with OS regarding the ability to induce osteogenic differentiation of MSC. Alizarin Red S stain was used on culture dishes (Figure 4) at days 14, 21, 28, 35 and 42 in order to monitor the progressive deposition of the mineral matrix. Cells started to produce a mineralized matrix on day 28, in both media, DRUGS and OS. Concomitantly, histologic analysis of the scaffolds were performed at 14, 21, 28 and 35 days of culture. Collagen sponges were frozen and sections were stained with H&E and with Alizarin Red S (Figure 5). Collagen scaffold

sections stained with H&E showed that MSC were able to distribute themselves uniformly between collagen meshes. Seeded MSC exposed to OS began the process of mineralization on day 21, while cells exposed to DRUGS started at day 28. MSC seeded on dishes and onto collagen scaffolds, and exposed to DRUGS, differentiated slower than MSC exposed to OS, as shown by Alizarin Red S stain. Even though DRUGS induction proved to be slightly weaker than OS, a sufficient level of deposited mineralized matrix was still observed after a longer culture time. The cell number was also determined at different time points and, even though MSC exposed to OS revealed higher cell numbers at 35 days, such a difference was not significant compared with DRUGS or untreated cells (data not shown).
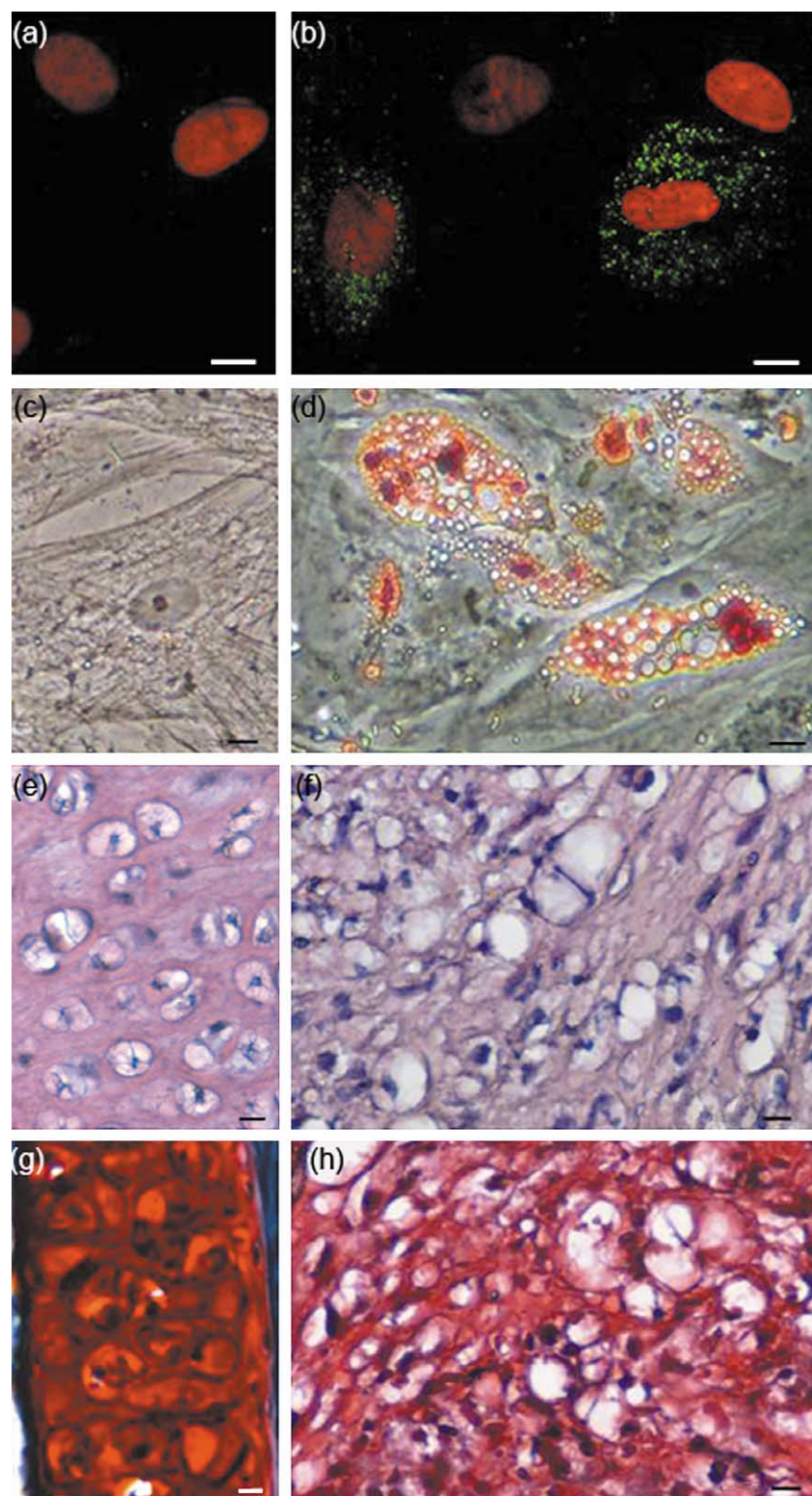
Figure 3. *MSC in vitro differentiation. (a, b) Osteogenic differentiation; osteopontin, evaluated by immunofluorescence, is expressed in treated cells (b) but not in untreated cells (a) (osteopontin, green; nuclei, red). (c, d) Adipogenic differentiation; Oil red O-stained lipid droplets are present in treated cells (d) but not in untreated cells (c). (e—h) Chondrogenic differentiation; H&E staining (e, f) reveals a similar morphology between treated cells (f) and ear cartilage (e) used as a positive control. (g, h) Safranin O staining of the extracellular matrix shows the presence of proteoglycans and glycosaminoglycans both in treated cells (h) and positive control (g). Bar = 10 μm.*
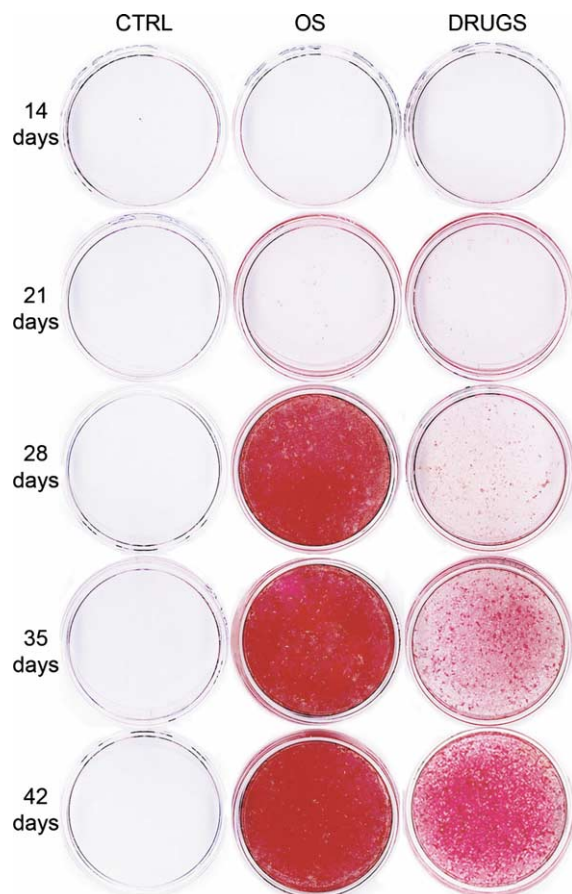
Figure 4. *Osteogenic differentiation of MSC. The figure shows Alizarin Red S stain of untreated cells (CTRL) and cells treated with standard osteogenic supplements (OS) or with clinical grade drugs (DRUGS) at different time-points after initial seeding. One representative experiment is shown (out of 4 performed).*

These data were repeated with cells from three other cell cultures and we always observed a weaker mineralization with DRUGS than OS. Between cells of the four different donors, the beginning of the mineralization process varied from day 21 to day 28. The results showed that our protocol of osteogenic induction of MSC seeded on scaffolds after exposure to clinical grade drugs is capable of producing osteogenic-differentiated MSC that distribute uniformly between collagen meshes.

Microbiologic analysis

An essential parameter of quality certification of the cell product is confirmation of its sterility, especially considering that MSC need long-term cultures and frequent manipulations. Tests for endotoxin, mycoplasma, aerobic bacteria, anaerobic bacteria and fungi were always negative.

Discussion

Our findings show that, in the specific context of a degenerative disease of the bone (periodontal disease) with limited involvement of the skeletal tissue, the combined use of MSC exposed to an osteogenic clinical grade medium and biomimetic biodegradable scaffolds can offer the possibility of producing adequate numbers of biologic tissue-engineered cell-based constructs to be used in clinical trials, with the aim of repairing distinct areas of bone resorption for the treatment of periodontal disease. Periodontal disease is a chronic and degenerative disease that eventually leads to the destruction of the periodontal apparatus, with resorption of the alveolar bone, the periodontal ligament, cementum and gingival mucosa, and the inevitable loss of the involved teeth [1,2]. New techniques of tissue engineering, such as the manufacture of synthetic materials and cell therapy, are under constant investigation, with the aim of generating clinical grade products capable of reconstituting the lost bone tissue.

Cell therapy has recently gained increasing interest regarding alternative approaches for tissue repair. In fact, the use of cell products offers various potential advantages compared with standard treatments, particularly in the context of degenerative diseases, because of the multipotent capacity of particular types of cells with staminal characteristics and the theoretical ability of such cells to recreate a damaged tissue when implanted under appropriate conditions [17]. However, clinical applications of cell therapies represent a complicated challenge, because of the necessity of operating under strict GMP conditions and the absolute need to demonstrate not just the safety of the proposed approach but, first and foremost, its feasibility and an acceptable degree of reproducibility for clinical applications [39].

The aim of our study was to validate, under GMP conditions, a method for obtaining a certified product of osteo-differentiated MSC placed on collagen sterile scaffolds to be implanted in areas of bone resorption in patients affected by severe forms of periodontal disease. The first goal of a successful clinical grade approach is to identify the most suitable cell population to start with, and the easiest way of obtaining a sufficient cell number without compromising the safety for the patient or rendering the approach too invasive. For these reasons we chose to employ autologous MSC isolated from BM aspirates [8–11]. The multipotent capacity of MSC, the possibility of easily isolating them from BM aspirates and
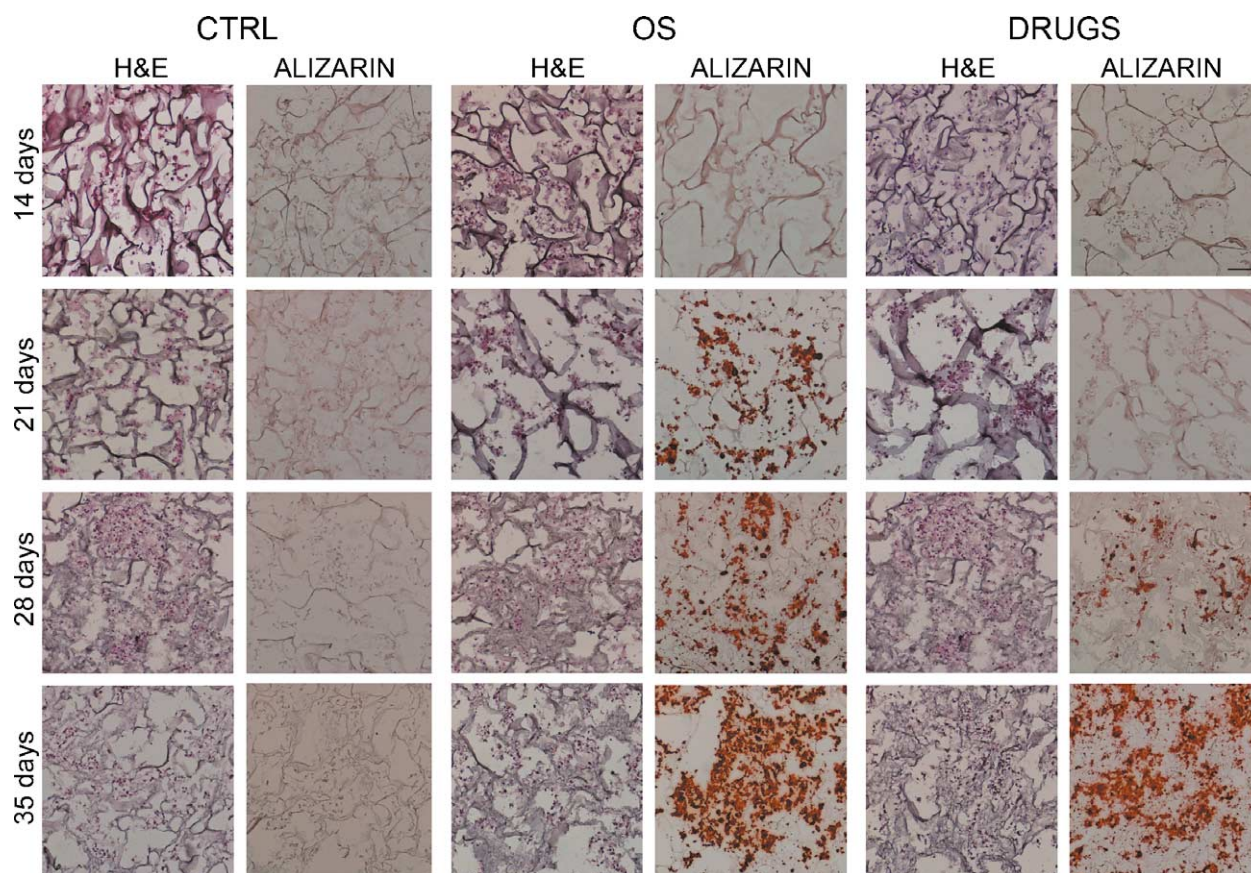
Figure 5. Osteogenic differentiation of MSC seeded on a collagen scaffold and exposed to CTRL, OS or DRUGS media. At 14, 21, 25 and 35 days, scaffolds were frozen and serial sections were stained with H&E or Alizarin Red S (ALIZARIN). One representative experiment is shown (out of 4 performed). Bar = 100 μm.

their high *ex vivo*-expansive potential [14,15], make these cells among the best candidates for cell therapy to regenerate injured skeletal tissues. Our results demonstrate that a limited amount of BM is sufficient to obtain adequate numbers of MSC to be subsequently differentiated in osteogenic precursors.

Once a sufficient number of putative MSC has been obtained, the precise characterization of this population represents a critical step before proceeding with the differentiation process. Even though the precise identification of unique markers to categorize the true mesenchymal subset is still a matter of investigation [40], nevertheless the high surface expression of CD105, CD90 and HLA-ABC combined with the absence of hematopoietic markers (CD33, CD34, HLA-DR) may represent a valid mean of MSC characterization [11,41]. In any case, our functional experiments demonstrate that MSC, when treated with specific induction media, were able to differentiate *in vitro* into osteogenic, adipogenic and chondrogenic lineages.

The final step is represented by the establishment of a system for creating a suitable environment to promote tissue growth and cell differentiation under GMP conditions. The first need was to identify a clinical grade cocktail of certified drugs that was equally functional compared with experimental reagents. While the use of the clinical grade cocktail requires longer time before producing deposition of a mineralized matrix (around 1 week more), the Alizarin Red stain consistently revealed a uniform presence of the mineralized matrix in all tested donors. Precise explanations for this discrepancy have not yet been identified. Our hypothesis is that this slower deposition could be because the ascorbic acid used in the clinical drugs cocktail is not as chemically stable as the 2P-ascorbic acid form used in the standard osteogenic supplements [42].

Cells also need an appropriate three-dimensional support to sustain their growth, uniform distribution and optimal conditions for differentiation after the inoculation

[43–45]. We chose to use the commercial clinical grade scaffold Gingistat and 1 million cells were seeded onto each 125-mm^3 scaffold, according to established published models [23,30,46,47]. Gingistat is already used as a hemostatic filling scaffold in periodontal disease [48]. In published experiments [34], we have shown that rat MSC are easily adsorbed and homogeneously distributed to the entire scaffold without losing their osteogenic differentiation potential. Although in our previous *in vitro* experiments this material proved to be biodegradable, with a linear trend in 5 weeks in the absence of cells, our current experiments have demonstrated that, in the presence of differentiated cells, the integrity of the scaffold can be preserved for at least 35 days.

Gingistat scaffolds can offer different advantages when applied to the correction of small bone defects, especially in the oral cavity. This sponge is manufactured for disposable sterile use, easily manageable in a laminar-flow cabinet and for surgical use, soft and therefore adaptable to different shaped areas. Moreover, various numbers of sponges can be produced and simultaneously placed to fill defects of larger depth.

We decided to induce *in vitro* MSC differentiation on the scaffold in line with several studies that have shown a marked improvement in terms of functional bone healing and rapidity of recovery [28–30]. This choice also allowed for a stricter control of quality parameters, which are necessary to establish the acceptability of the cell product before its implant. In this way we were able to detect the beginning of the mineralization process and therefore establish a suitable moment to plan the surgical placement of the scaffold. Although a recent study by Niemeyer *et al.* [49] shows the equivalency of this *in vitro* manipulation with the implant of unmanipulated MSC onto a mineralized collagen scaffold, nevertheless, in our opinion, an *ex vivo* differentiation allows a better definition of quality parameters and, therefore, a more adequate compliance to GMP-grade methodology.

All these data suggest the feasibility of using MSC and Gingistat scaffolds in a clinical protocol that envisages the use, under GMP conditions, of collagen scaffolds with MSC exposed to a clinical grade osteogenic medium, being implanted into periodontal lesions with the aim of repairing alveolar bone defects. Our clinical trial will firstly aim to demonstrate the feasibility of our proposed system for producing certified, sterile cell products in sufficient numbers to treat bone defects of different size and depth, and, secondly, to verify the absence of toxicity, even though little or none is expected because of the simplicity of the surgical procedure, which is represented by a local delivery of soft biodegradable materials. We do not predict any immune side-effect related to the use of FBS, the clinical trial being based on the administration of a single MSC infusion. Only one clinical episode of immune reaction, related to FBS, has been described in the literature and it was related to multiple systemic administrations after intravenous injections [50]. If any future impediment should occur, further improvements of cell production can be achieved by use of serum-free media, recently tested for GMP-grade MSC expansion [51]. It is difficult to predict what impact this product will have in terms of true bone formation following implant, but we can certainly hypothesize that the presence of inflammatory factors at the site of periodontal disease may partially promote the process of bone deposition, once the initial step has been primarily induced *in vitro*. The data reported by the Cancedda group [32] regarding the correction of large bone defects and the use of macro-porous hydroxyapatite scaffolds show that an abundant callus formation can be observed and good integration of the construct achieved at the interface with surrounding host bone, accompanied by a clear clinical improvement of limb functions in a shorter time than traditional approaches. Similar results are envisaged in our trial, despite treating different bone defects and using a different way of producing osteo-differentiated MSC and a different type of scaffold.

In conclusion, our results show that BM-derived MSC can be manipulated *ex vivo* under GMP conditions without compromising their functional properties, to obtain finally a cell-based tissue-engineered product of biodegradable soft sterile collagen scaffolds to support and induce the repair of bone loss in patients affected by severe forms of periodontal disease.

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