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The recruitment of two consecutive and different waves of host stem/progenitor cells during the development of tissue-engineered bone in a murine model

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ABSTRACT

Angiogenesis plays a central role in bone regeneration, not only for the transport of nutrients, but also for locally directing skeletal stem/progenitor cells. Following ectopic implantation of porous ceramic cubes seeded with mouse GFP-labeled mesenchymal stem cells (MSC) into syngenic mice, we investigated the cascade of events leading to bone formation. Implants harvested at different times were enzymatically digested to generate single-cell suspensions. Recovered cells were sorted to separate GFP + implanted MSC and host recruited GFP- cells. We isolated and characterized two different waves of cells, migrating from the host to the MSC-seeded ceramic. The first migrated cell population, recovered 7 days after implantation, was enriched in CD31 + endothelial progenitors, while the second one, recruited at day 11, was enriched in CD146 + pericyte-like cells. Both populations were not recruited into the scaffold following implantation of a non-MSC seeded ceramic. Pericyte-like cell mobilization was dependent on the first migrated endothelial cell population. Pericyte-like cells retained properties distinctive of stem cells, such as capacity of performing a high number of *in vitro* cell divisions and showed an osteogenic potential. Studies on the cross talk between implanted exogenous MSC and resident stem/progenitor cells could open new perspectives for future clinical applications.

1. Introduction

Bone is a highly vascularized tissue in which the close connection between blood vessels and bone cells maintain skeletal homeostasis. Angiogenesis plays a critical role in skeletal development and bone fracture repair. Indeed, within specialized niches, bone formation is mediated by the interplay of hematopoietic, endothelial, immune cells, and bone marrow precursors, including stem cells. The importance of endothelial cells in bone homeostasis has been confirmed by recent publications where the mobilization of circulating bone marrow-derived endothelial progenitors to sites of tissue regeneration has been described [1–3]. Moreover, it has been demonstrated that these cells can participate to the neovascularization processes [4–7].

The development of vessel-like structures is the first specific reaction of an organism to an organ graft [8–10]. Today, tissue engineering offers valid alternative therapeutic strategies to support tissue healing. In the case of transplantation of large engineered tissue implants, such as transplantation of cell-seeded ceramic scaffolds to repair large bone defects, the vascularization of the implant represents a very critical step, not only for the *in vivo* engraftment and integration of the engineered tissue [11], but also for the mobilization of cells of host origin, creating a communicative network between the recipient organism and the engineered graft.

Differentiated microvessels are characterized in large part by the association of endothelial cells with pericytes, and failure of these interactions results in severe, and often lethal, defects [12]. Vascular endothelial cells respond to stimuli of different nature, such as growth factors, cytokines, and lipoproteins [13]. Studies on the control of vasculogenesis in the human dermis [14] indicate that mesenchymal stem cells (MSC) can function as vascular pericytes [15,16]. These cells are present in multiple human organs, where they are characterized by the expression of specific cell surface antigens, such as Melanoma-associated Cell Adhesion Molecule (MCAM/CD146), the proteoglycan NG2, Platelet Derived Growth Factor-Receptor α (PDGF-Rα), and by the lack of endothelial and myeloid markers [17]. In addition to participate in the maintenance of blood vessel wall integrity, $CD146^{+}$ sub-endothelial

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pericytes retain the pluripotency typical of MSC [16], other than their immunomodulatory and trophic properties [15].

Recent literature reports indicate that the mobilization of endogenous cells into the site of the lesion can mediate tissue repair [18–20]. We recently published that the ectopic implantation of ceramic scaffolds seeded with murine Green Fluorescent Protein-positive (GFP^+) MSC into immunocompromised and immunocompetent syngenic recipients resulted in the formation of a host-derived engineered bone tissue within the pores of the scaffolds after 60 days of *in vivo* implantation [9,21].

Here, we implanted combinations of GFP^+ MSC and porous ceramic scaffolds in immunocompetent syngenic recipients to study the cascade of events leading to the development of the tissue-engineered bone, as well as the characteristics of the endogenous cells recruited within the implants. This approach allowed us to isolate and characterize two waves of cells of different nature actively participating in the formation of the newly-formed bone and specifically migrating from the host to the implanted graft only when the ceramic was seeded with exogenous MSC.

2. Materials and methods

2.1. Mice

C57Bl/6 (MHC $H2^b$ haplotype), Balb/c (MHC $H2^d$ haplotype), and Swiss Nude (CD1 nu/nu, outbred strain) mice were purchased from Charles River Laboratories (Calco, LC, Italy). Green Fluorescent Protein (GFP)-transgenic mice (GFP-Tg) (genotype C57Bl/6-transgenic (ACTB-EGFP) 1Osb/J mice, MHC $H2^b$) were purchased from The Jackson Laboratory (Bar Harbor, MA, USA). Mice were used between five and eight weeks of age. Mice were bred and maintained at the Institution's animal facility. The care and use of the animals were in compliance with the laws of the Italian Ministry of Health and the guidelines of the European Community.

2.2. Cells

MSC were obtained from GFP-Tg mice. Bone marrow cells were collected by flushing nucleated cells out of the femurs and tibiae with cold phosphate-buffered saline (PBS). Cells were cultured (10×10^6 nucleated cells/10 cm Petri dish) in Coon's modified F12 medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (GIBCO, S. Giuliano Milanese, MI, Italy), 2 mM L-glutamine, 50 mg/ml penicillin/streptomycin and, unless otherwise indicated, also containing 1 ng/ml basic-fibroblast growth factor (FGF-2) (standard medium). MSC were allowed to adhere to the plastic support of the Petri dish for 3 days before the first medium change. All cultures were maintained in a humidified incubator at 37 °C with 5% CO_2. Only cells that were in passage 1 or 2 were used for the implantation experiments.

Spleens from Balb/c mice were collected after euthanasia. Spleen cells were obtained by mechanical shredding and collected in RPMI-1640 medium (Sigma, Milano, Italy) supplemented with 2-mercaptoethanol (2-ME), glutamine, nonessential amino acids, sodium pyruvate, antibiotics (Sigma, Milano, Italy) and 1% normal mouse serum. Spleen cells were used immediately after isolation.

Ectopic MSC/bioscaffold constructs implanted in syngenic mice were harvested 3, 7, and 11 days after implantation. We carefully removed the uncalcified surrounding tissues from the constructs, and we cut the constructs in two parts under sterile conditions. The implants were washed in HBSS/30 mM HEPES (Euroclone, MI, Italy), and digested twice with 100U/ml type II Collagenase (Biochrom AG, Berlin, Germany) in PBS/2.5% trypsin (Gibco, MI, Italy) for 40 min at 37 °C. Cell suspensions derived from these serial digestions were subsequently used for cell sorting in order to separate and quantify GFP^+ implanted cells and GFP^- cells recruited within the implants. Aliquots of GFP^- recruited cells were plated and expanded in culture in standard medium on fibronectin-coated 6-well plates (Becton Dickinson, Bedford, MA, USA).

2.3. Surgical procedures

In vivo transplantation of GFP^+ MSC in syngenic recipient mice was performed as previously reported [9]. Briefly, 2.5×10^6 cells were seeded onto a highly porous ceramic support based on a 100% hydroxyapatite (HA) scaffold ("EngiPore"-EP) (FinCeramica, Faenza, Italy) ($4 \times 4 \times 4$ mm cubes), before being embedded in a fibrin gel. The scaffolds present a porosity level of approximately 70–80% and the following pore size distribution: <10 μm, ~3% vol; 10–150 μm, ~11% vol; >150 μm, ~86% vol.

Scaffolds seeded with GFP^+ MSC and non-seeded empty scaffolds, used as control, were subcutaneously implanted into 16 syngenic mice. Groups of four animals were sacrificed after 3, 7, and 11 days post-implantation, and the implants removed for further analysis.

Additional *in vivo* experiments were performed implanting 6 Swiss Nude mice and 3 C57Bl/6 mice with HA cubes seeded with Double Positive (DP) and Double Negative (DN) cells, recruited after 7 and 11 days (for DP and DN definition, see "Immunomagnetic separations" paragraph in the "Materials and Methods" section). These implants were extracted 8 weeks after implantation for histological analysis.

2.4. Cell sorting

As previously described, freshly isolated cells obtained from serial enzymatic digestions of MSC-seeded scaffolds ectopically implanted in syngenic mice, were harvested 3, 7 and 11 days after implantation. Cells were washed, suspended in 500 μl PBS, and separated into GFP^- and GFP^+ fractions using the cell sorter FACSAria (Becton Dickinson, Bedford, MA, USA).

2.5. Immunomagnetic separations

Host-derived GFP^- cells, sorted 7 and 11 days after implantation of MSC-seeded scaffolds in syngenic recipients, were immunomagnetically separated in two fractions, the $CD14^+CD45^+$ double positive (DP) subpopulation and the $CD14^-CD45^-$ double negative (DN) subpopulation. To obtain DN and DP populations of GFP^- recruited cells, we first performed a CD14 enrichment using the mini-MACS immunomagnetic separation system following the manufacture's instructions (Miltenyi Biotec, Bergish Galdbach, Germany) and the monoclonal antibody rat anti-mouse CD14 (clone: rmC5-3) (BD PharMingen, MI, Italy). We obtained a $CD14^-$ and a $CD14^+$ fraction. Then we performed the CD45 enrichment in these last two populations using the mini-MACS immunomagnetic separation system following the manufacture's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) and the monoclonal antibody rat anti-mouse CD45 (clone: 104) (BD PharMingen, MI, Italy).

2.6. Phenotype of cell populations

MSC cultured in standard medium either in presence and absence of basic-Fibroblast Growth Factor (FGF-2) were analyzed for the expression of the markers CD146 (clone P1H12) (Santa Cruz Biotechnology, Heidelberg, Germany), CD117 (clone 3C1) (Miltenyi Biotec, Bergish Galdbach, Germany), CD140a (PDGF-Rα) (clone APA5) (BD Pharmingen, MI, Italy), and CD31 (Santa Cruz Biotechnology, Heidelberg, Germany).

Immunophenotype of freshly-isolated DP and DN cells recruited 7 and 11 days after implantation, immunophenotype of cultured DN cells recruited 7 and 11 days after implantation, and immunophenotype of cells recruited 7 and 11 days after implantation of empty scaffolds, were analyzed by flowcytometry using monoclonal antibodies to CD4, CD11a, CD14, CD31, CD34, CD44, CD45, CD90, CD106 (clones GK1.5, M1/70, rmC5-3, RAM34, M7, 104, 30-H12, 429) (BD PharMingen, MI, Italy), to CD117 (clone 3C1) (Miltenyi Biotec, Bergish Galdbach, Germany), to CD146 (clone P1H12) (Santa Cruz Biotechnology, Heidelberg, Germany), and to polyclonal CD31 (Santa Cruz Biotechnology, Heidelberg, Germany).

Immunophenotype of freshly-isolated cells, extracted 60 days after implantation of DN-11 cells/bioscaffolds, was analyzed by flowcytometry using a monoclonal antibody to $H\text{-}2K^b$ (clone AF6-88.5) and a polyclonal antibody to osteocalcin (AbD Serotec, Oxford, UK).

2.7. Histological analysis

Formalin-fixed HA blocks were processed as reported [9]. Briefly, samples were decalcified with Osteodec (Bio-Optica, Milano, Italy) and embedded in paraffin using standard histological techniques. Four-micrometer serial sections were cut. Sections were stained with hematoxylin and eosin (H&E) to reveal bone deposition.

2.8. Growth kinetics determination

In vitro proliferation rate and differentiation potential of DN and DP cells recruited 7 and 11 days after implantation were studied on cells cultured in fibronectin-coated plates. Cells were plated at 5×10^4 cells/6 cm dish in triplicate. When the plated cells reached about 80% confluence they were trypsinized and replated in new dishes at the same initial concentration. At each passage, the number of doublings was calculated according to the formula: Log2 of cells obtained/cells plated (at each passage) plotted against time in culture.

2.9. Matrigel morphogenesis assay

The ability of freshly isolated and of cultured (passage 7) DN and DP cells, extracted from 7 and 11 day-implants, to reorganize and differentiate into tubular networks was assessed in the Matrigel morphogenesis assay. Matrigel (BD Biosciences, Bedford, MA, USA) (300 μl/well) thawed at 4 °C in an ice-water bath was carefully added with a cold pipette to a pre-chilled 24-well plate. After polymerization of Matrigel at 37 °C for 30 min, 7×10^4 cells/well were layered in Coon's F12 medium either in presence and absence of serum on top of the polymerized gel. The