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Enhanced human bone marrow mesenchymal stromal cell adhesion on scaffolds promotes cell survival and bone formation

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ABSTRACT

In order to induce an efficient bone formation with human bone marrow mesenchymal stromal cells (hBMSC) associated to a scaffold, it is crucial to determine the key points of the hBMSC action after *in vivo* transplantation as well as the appropriate features of a scaffold. To this aim we compared the hBMSC behavior when grafted onto two biomaterials allowing different bone potential *in vivo*. The cancellous devitalized Tutoplast®-processed bone (TPB) and the synthetic hydroxyapatite/β-tricalcium-phosphate (HA/βTCP) which give at 6 weeks 100% and 50% of bone formation respectively. We first showed that hBMSC adhesion is two times favored on TPB *in vitro* and *in vivo* compared to HA/βTCP. Biomaterial structure analysis indicated that the better cell adhesion on TPB is associated to its higher and smooth open pore architecture as well as its content in collagen. Our 6 week time course analysis, showed using qPCR that only adherent cells are able to survive *in vivo* giving thus an advantage in term of cell number on TPB during the first 4 weeks after graft. We then showed that grafted hBMSC survival is crucial as cells participate directly to bone formation and play a paracrine action via the secretion of hIGF1 and hRANKL which are known to regulate the bone formation and resorption pathways respectively. Altogether our results point out the importance of developing a smooth and open pore scaffold to optimize hBMSC adhesion and ensure cell survival *in vivo* as it is a prerequisite to potentiate their direct and paracrine functions.

Statement of Significance

Around 10% of skeletal fractures do not heal correctly causing nonunion. An approach involving mesenchymal stromal cells (MSC) associated with biomaterials emerges as an innovative strategy for bone repair. The diversity of scaffolds is a source of heterogeneity for bone formation efficiency. In order to better determine the characteristics of a powerful scaffold it is crucial to understand their relationship with cells after graft. Our results highlight that a biomaterial architecture similar to cancellous bone is important to promote MSC adhesion and ensure cell survival *in vivo*. Additionally, we demonstrated that the grafted MSC play a direct role coupled to a paracrine effect to enhance bone formation and that both of those roles are governed by the used scaffold.

1. Introduction

Bone has the particularity to regenerate after a lesion allowing fracture repair in 6–8 weeks [1]. Bone regeneration is mostly spontaneous and surgery occurs only to mechanically stabilize the fracture [2]. However, 5 to 10% of skeletal fractures do not heal correctly causing delayed union or nonunion called pseudarthrosis

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[3]. These defects require additional interventions with a major impact on patient life quality and with socio-economic burden [2,3]. To manage this problem, different therapeutic strategies have been developed. Autologous cancellous bone graft is still until today the gold standard for regeneration of bone defects exceeding 4 cm [2]. Although this bone transplantation approach has shown its clinical efficacy, it presents several drawbacks (i.e. limited bone stock, additional surgical site with consequent pain and risk of infections) [4]. Other approaches have been developed for clinical use. Allogeneic bone graft (i.e. surgical waste, post-mortem donors) is an alternative to autograft [5]. Bone xenograft has also been used, such as bovine bone whose structure is close to humans [6]. However, these strategies may transfer pathogens [7] or lead to immunological reactions [8]. To overcome bone graft disadvantages, several biocompatible scaffolds have been developed [9–11]. Synthetic bioceramics of hydroxyapatite (HA); tricalcium phosphate (TCP) or their mixture biphasic calcium phosphate (BCP) are the most used in skeletal engineering because of their osteoconductive properties and the easy management of their architecture [12]. Alternative natural scaffolds including secure amorphous human bone [9,10], animal derived bone matrices [13] or the coral exoskeleton [11] offer a similar structure to cancellous bone and thus mimic the physiological bone tissue environment [12]. However, when used alone these scaffolds lack osteoinductive properties and serve only to guide bone tissue [4]. To enhance new bone formation, growth factors (FGF-2, PDGFs) or differentiating factors (BMPs) and more recently stem cells have been associated to these scaffolds [3,14,15]. An approach involving human bone marrow mesenchymal stromal cells (hBMSC) associated with biomaterials emerges as an innovative strategy to repair delayed unions [11]. Mesenchymal stromal cells (MSC) ability to differentiate into several tissues including bone, cartilage and adipose [16] was widely exploited for tissue regeneration. While their capacity to induce bone formation has been proven, mechanisms of fracture repair remain controversial. Several studies suggest a direct contribution of MSC to regenerate bone as they differentiate into osteoblastic lineage when implanted locally or even when administrated systemically [17,18,23]. Ponte et al., suggest that the host inflammatory environment at the injury site induces the recruitment and the engraftment of MSC [19]. This homing can be under the control of SDF-1/CXCR-4 signaling [20,21] but also mediated by other cytokines such as IGF-1 and PDGF [19]. Nonetheless, divergent studies are in favor of a paracrine effect of grafted MSC on host cells *via* the release of cytokines and growth factors. In response to inflammation at the bone lesion site, grafted MSC secrete immunomodulatory cytokines such as prostaglandin 2, TGF-β1, TNF-α, IL-4, 6 and 10 to prevent proliferation of inflammatory cells including T and B lymphocytes, natural killer cells and macrophages [22,23]. In addition, secretion of factors such as SDF-1 and macrophage inflammatory protein-1 α and β reduce scar tissue formation [22]. MSC are also able to initiate angiogenesis and enhance endothelial cells recruitment and proliferation through secreted growth factors including VEGF, IGF-1, angiopoietin-1 and EGF [24]. It has also been shown that hBMSC recruit macrophages and osteoclasts at the site of implantation prior to bone formation [25]. In summary, MSC ability to differentiate in bone lineage, associated to their paracrine functions and their availability, make them a promising candidate in regenerative medicine [22]. Hence, these properties were clinically exploited to develop a minimally invasive therapeutic strategy for bone repair [26–28].

Our team demonstrated that hBMSC expansion e*x vivo* using human platelet lysate accelerates their proliferation and prime their osteogenic differentiation [29]. This strategy allows the use of a growth factors cocktail (e.g. BMPs, TGF-b1, IGF, bFGF, PDGF, PF-4, interleukin-1, and osteonectin) to stimulate multiple signaling pathways, as opposed to previous strategies where a single recombinant growth factor was used and acted only on one molecular pathway [29,30]. Moreover, we and other observed a variability in the osteoconductive and osteoinductive properties of different bone allografts or ceramics resulted in differences in *in vivo* bone formation [10,31]. Cell attachment and metabolism vary between scaffolds having the same composition but with different structures [31,32] demonstrating that the scaffold geometry is crucial to guide bone formation [33].

In order to induce an efficient bone formation, it is crucial to determine the key points of the hBMSC action after graft *in vivo* as well as to determine the main characteristic of a powerful scaffold. To this aim we followed and compared during a 6 week time course, the *in vivo* hBMSC behavior when grafted onto two different biomaterials allowing different bone potential. To this end, the cancellous devitalized Tutoplast®-processed bone (TPB) and the synthetic hydroxyapatite/β-tricalcium-phosphate (HA/βTCP) which are clinically used as bone substitutes [9,29,31,34]. Human BMSC were collected from several donors, amplified *in vitro* and then loaded extemporaneously on scaffolds implanted subcutaneously in SCID mice, as this model is more efficient to induce *in vivo* bone formation [34]. After having evaluated the biomaterial impact on ectopic bone formation, we evaluated during a 6 week time course, their impact on cell attachment and survival, on cell dissemination as well as on hBMSC behavior and function. We then performed *in vitro* analysis in order to discriminate the direct relationship between the scaffold structure and the *in vivo* cell feature.

2. Materials and methods

2.1. Biomaterials

Two scaffolds with different structure and composition were used. (1) Hydroxyapatite (65%)/beta-tricalcium phosphate (35%) (HA/βTCP) (Ceraver, France) is composed of 60 ± 5% macropores (100 to 400 μm pore diameter) and 40 ± 5% micropores (<10 μm pore diameter). (2) Tutoplast®-processed human bone (TPB) (EFS, Ile de France). The bone Tutoplast®-process (Tutogen Medical) consists in a delipidization, an osmotic cell destruction treatment, hydrogen peroxide treatment, and washing cycles for the removal of the noncollagen proteins followed by a solvent dehydrated step and finally a γ-irradiation procedure. Both biomaterials were in the form of irregular granules sizing 2–3 mm and weighing 8.0 ± 1 mg.

2.2. Physico-chemical characterization of biomaterials

The microstructure of the scaffolds was investigated by using a scanning electron microscope (SEM; Hitachi TM3000 Tabletop Microscope, Tokyo, Japan). Prior to SEM observations, the samples were coated with a thin film of carbon obtained by glow discharge (Leica, ACE 200, Germany). Powder X-ray diffraction (XRD, Siemens D5000 Moxtek) was performed on crushed scaffolds to determine the phases and crystallinity using the monochromatic source KαCu. Fourier Transform Infrared Spectroscopy (FTIR, Bruker, Vertex 70) was performed on KBr pellets with 1 mg of sample mixed with 300 mg of KBr and pressed at 5 tons. Micro-computed tomography (μCT, Skyscan 1076 *in vivo* model, Kontich, Belgium) of scaffolds was performed. Briefly, the X-ray source was operated at 50 kV and acquisitions were recorded around 180° with step angle of 0.7°. Three dimensional (3D) reconstructions were performed using the Skyscan software CTRecon. The morphometric parameters, Percent Object Volume/Total Volume (Obj.V/TV, %), Object Surface (Obj.S, mm^2) and Open Porosity (%) of both scaffolds were measured by using the Skyscan™ CT-ANalyzer software (n = 5 samples/biomaterial scaffold).