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The effect of erythropoietin on autologous stem cell-mediated bone regeneration

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ABSTRACT

Mesenchymal stem cells (MSCs) although used for bone tissue engineering are limited by the requirement of isolation and culture prior to transplantation. Our recent studies have shown that biomaterial implants can be engineered to facilitate the recruitment of MSCs. In this study, we explore the ability of these implants to direct the recruitment and the differentiation of MSCs in the setting of a bone defect. We initially determined that both stromal derived factor-1alpha (SDF-1α) and erythropoietin (Epo) prompted different degrees of MSC recruitment. Additionally, we found that Epo and bone morphogenetic protein-2 (BMP-2), but not SDF-1α, triggered the osteogenic differentiation of MSCs *in vitro*. We then investigated the possibility of directing autologous MSC-mediated bone regeneration using a murine calvaria model. Consistent with our *in vitro* observations, Epo-releasing scaffolds were found to be more potent in bridging the defect than BMP-2 loaded scaffolds, as determined by computed tomography (CT) scanning, fluorescent imaging and histological analyses. These results demonstrate the tremendous potential, directing the recruitment and differentiation of autologous MSCs has in the field of tissue regeneration.

1. Introduction

A number of tissue regeneration applications have substantially improved over the years with the advent of stem cell technologies. Over the years, tissue engineering has emerged as a promising alternative with remarkable progress being made in understanding the role played by mesenchymal stem cells (MSC), scaffolds, growth factors and bioreactors [1]. MSCs are of great importance for regeneration of tissues of the mesenchyme, like bone [2]. Taking advantage of their potent tissue regenerative properties, MSCs have been employed extensively as the cell source for bone regeneration [3–7]. Almost all of these studies relied on the isolation, culture and seeding of MSCs onto scaffolds prior to implantation [8–11]. They have shown that the presence of MSCs substantially enhance the extent of mineralized bone regeneration [9–11]. Despite these impressive outcomes, the use of MSC-seeded scaffolds in a clinical setting, such as in the reconstruction of critical size bone defects, has been limited. Limiting the usefulness of these applications is the need to isolate and culture MSCs from the patients, which is, time consuming, expensive and cannot be mass produced [12,13]. Therefore, there is a need for further development of new technologies for bone tissue engineering employing autologous MSCs.

It is well established that, shortly after fracture, MSCs are recruited to the injured site and differentiate into osteoblasts prior to bone regeneration. We hypothesized that bone regeneration can be achieved by recruiting and causing the differentiation of MSCs into osteoblasts with the use of scaffolds loaded with certain cytokines. Ideally, tissue regenerating scaffolds should be able to recruit and then cause the differentiation of MSCs. Ours and other groups' studies have shown that large numbers of MSCs and hematopoietic stem cells (HSCs) are recruited to the biomaterial implant sites [14,15]. The recruitment of MSCs is likely to be associated with the release of several inflammatory chemokines, including, granulocyte colony-stimulating factor, granulocyte macrophage-colony-stimulating factor, monocytes chemoattractant protein, macrophage inflammatory protein, matrix metalloproteinase-2, and tissue

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inhibitor of metalloproteinase-2 [16–18]. However, these inflammatory chemokines also promote the accumulation of inflammatory cells [19–22]. Our recent studies have shown that the localized release of SDF-1α enhances MSC recruitment while reducing inflammatory cell recruitment to the implant sites [23]. Interestingly, localized stromal derived factor-1α (SDF-1α) treatment was also found to increase angiogenesis around the scaffolds [23]. Erythropoietin (Epo) has recently been shown to be a potent chemokine for both MSCs and HSCs recruitment as well as angiogenesis [24–28]. As a result, these data have stimulated significant interest in the role osteogenic and angiogenic cytokines can play in bone tissue engineering applications [29,30]. Interestingly, a recent report has suggested that Epo triggers the differentiation of MSCs into osteoblasts [31]. Based on these reports and our own results, we have focused our investigation on the pro-osteogenic effects of SDF-1α and erythropoietin (Epo). In the past, bone morphogenetic protein-2 (BMP-2) has been used as an osteogenic agent in various clinical trials [32,33]. It has been well established that incorporation of BMP-2 in tissue engineering scaffolds promotes bone mineralization both *in vitro* and *in vivo* [32,33]. Therefore, in addition to the aforementioned individual chemokines, the effect of BMP-2 either alone, or in combination with Epo and SDF-1α was also investigated.

A series of studies were carried out to test the hypothesis that autologous stem cells can be recruited and caused to differentiate into bone forming cells in an effort to regenerate bone tissue. First, chemokines SDF-1α and Epo were tested specifically for their ability to recruit MSCs. To do this, these chemokines were loaded onto scaffolds produced using our established protein microbubble scaffold fabrication technique [34], and the ability of these scaffolds to recruit stem cells was assessed *in vivo*. We then evaluated the ability of MSC chemokines and known osteogenic differentiation agent BMP-2, to differentiate MSCs into bone *in vitro*. Scaffolds loaded with these chemokines were tested for their ability to elicit bone regeneration using a murine calvarial defect model.

2. Materials and methods

2.1. Materials

Poly (d, l-lactic-co-glycolic acid) PLGA (75:25) with a molecular weight of 113 kDa was purchased from Medisorb (Lakeshore Biomaterials, Birmingham, AL). The solvent 1, 4-dioxane was purchased from Aldrich (Milwaukee, WI), Gelatin from Sigma (St Louis, MO) and Masson's trichrome kit was purchased from Sigma (St Louis, MO). Primary antibodies (1:50) against CD105, CD146, Stro-1, CD45 and osteocalcin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while CD34 and CD56 antibodies were purchased from BD Biosciences Pharmingen (BD Biosciences, San Jose, CA). Secondary antibodies (1:100) labeled with FITC or Texas Red were from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). SDF-1α was purchased from Prospec-Tany TechnoGene Ltd (East Brunswick, NJ), Epo from Cell Sciences (Canton, MA) and BMP-2 from R&D Systems (Minneapolis, MN). Oyster800 used for scaffold drug release studies was purchased from Boca Scientific Inc., (Boca Raton, FL). All Balb/c mice used in this work were obtained from Taconic Farms (Germantown, NY) and were cared for in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Arlington.

2.2. Role of chemokines in MSC migration

A transwell model was employed to compare the chemotaxis properties of various chemokines [23]. Briefly, Epo (200 IU) based on our pilot studies, or SDF-1α (10 ng/ml) based on our earlier publication [23], was added in the lower chamber of 8 μM pore membranes of 24-well plates (Corning Costar, Corning, NY). BM-MSCs was isolated from femurs and then cultured based on established procedures [35]. Murine MSCs (SSEA4+/CD45-) (~50,000 cells/well) were seeded on to transwell inserts (six wells per group). After incubation for 8 h at 37 °C, the numbers of transmigrated cells were quantified under fluorescent microscope following nuclear staining with DAPI. BM-MSCs in media without any cytokine additives served as controls.

2.3. Tracking injected stem cells in vivo

Bone marrow MSC were isolated from Balb/c mice femurs as described earlier [23]. Cells from the second passage were incubated with 5 μM of near-infrared dye (X-sight, Rochester, NY) at 37 °C for 24 h. After washing the cells with PBS, cells were injected through the dorsal tail vein in Balb/c mice bearing microbubble (MB) scaffolds (see Supplement) loaded with cytokines SDF-1α or Epo. MB scaffolds without any cytokines served as control. There were four animals per group. After 24 and 48 h following implantation, the stem cell recruitment to the implants was determined using an *in vivo* imaging machine (Kodak Image System FX, excitation of 760 nm, emission of 830 nm, 45 s exposure, 8 × 8 binning, f-stop 2.5, field of view 120 mm) similar to our earlier studies [15,23].

2.3.1. In vivo autologous stem cell recruitment to chemokine loaded MB scaffolds

SDF-1α or Epo loaded MB scaffolds were implanted subcutaneously in Balb/c mice (four mice per group). At one week post-implantation, the scaffolds along with the surrounding tissues were explanted and histological analyses was performed to determine the presence of various stem cell surface markers (MSCs: CD105+CD45-CD34-CD56-; Multipotent stem cells: CD146+CD45-CD56-; Pre-osteogenic stem cells: Stro-1+CD45-CD56-). The positive markers were labeled red while the negative markers were labeled green. Cells that were exclusively red were quantified using ImageJ as described earlier [15].

2.4. In vitro osteogenic differentiation of MSC

The osteogenic potential of bone marrow derived MSCs (BM-MSCs) was assessed as described earlier [36]. BMP-2 (200 ng/ml) was used in addition to Epo and SDF-1α. Briefly, BM-MSCs (3000 cells/mm^2) were incubated with different growth factors/chemokines in the presence of osteogenic cocktail. After incubation (7 days), the numbers of variously treated cells (six wells per treatment) were quantified using MTS assay to assess the effect of treatment on cell growth [34,37]. After incubation (21 days), cell differentiation into an osteogenic lineage was determined using Alizarin Red staining for calcified deposits [36].

2.5. Calvarial defect in animal model

The animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas at Arlington. A calvarial defect model was created as published earlier (six Balb/c mice per group) [38,39]. Briefly, following anesthesia, a defect measuring approximately 5 mm in diameter was created in the cranium with a drill taking care to avoid damage to the dura in a fashion similar to earlier publications [29,30]. The periosteum surrounding the defect was left untouched. Different scaffolds were implanted into the defect prior to wound closure. Analyses of osteogenic activities at the defect sites were done at the end of 4 and 8 weeks. Systemic administration of antibiotics was not used as based on our earlier study, it could affect the stem cell responses at the implant site [15].

2.6. CT scan

After 8 weeks, cranial explants were examined with a Siemens Inveon CT/PET Multimodality system (Siemens Medical Solutions Inc., Knoxville, TN, USA) operating in the cone-beam method. Images were obtained at 80 kV and 500 mA with a focal spot of 58 μm. The total rotation of the gantry was 360° with 1080 rotation steps obtained at an exposure time of approximately 715 ms/frame. The images were attained using a bin factor of 1 and an average frame of 3. Under high magnification the effective pixel size was 11.34 μm. CT images were reconstructed with a down-sample factor of 1 using Cobra Reconstruction software. For each sample, 1760 tomographic slices were obtained. Three-dimensional reconstruction of the bone was analyzed with the manufacturer's software. For each cranial bone sample, regions of interest (ROI) were drawn in the defect area. Fewer contours needed to be drawn since a routine facility calculated all the intermediary masks by interpolation. The ROI's were interpolated yielding a final ROI encompassing approximately 5 slices. Bone volume fraction (BV/TV), which is the ratio of total bone volume (BV) to total volume (TV, whole defect area), was the parameter used to determine bone growth.

2.7. Histological evaluation of osteogenesis

The implants along with the surrounding tissues were embedded for histological evaluation at 1, 4 and 8 weeks. To assess the influence of various chemokines/growth factors on stem cell responses, we quantified the numbers of MSCs and pro-osteogenic stem cells in implants/surrounding tissues using their unique sets of markers. To investigate the effect of scaffold implants on osteoblast differentiation, we evaluated the expression of osteocalcin and osteopontin (osteoblast products) in scaffold implant and surrounding tissue. Some tissue sections were stained with Masson's Trichrome blue for collagen in which nuclei stains blue-black and collagen stains blue. Stained sections were imaged using a Leica fluorescence microscope (Leica Microsystems, Wetzlar, GmbH) equipped with a CCD Camera (Retiga EXi, QImage). The fibrous tissue thickness was measured and quantified based on H&E staining. The area fraction of the Masson's Trichrome Blue stained sections was quantified using ImageJ to determine collagen coverage similar to earlier publications [34]. The fluorescence intensity of the immunofluorescence stained images was determined using "integrated density" feature in ImageJ under Analyze – Set