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Effect of copper-doped silicate 13–93 bioactive glass scaffolds on the response of MC3T3-E1 cells in vitro and on bone regeneration and angiogenesis in rat calvarial defects in vivo

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ABSTRACT

The release of inorganic ions from biomaterials could provide an alternative approach to the use of growth factors for improving tissue healing. In the present study, the release of copper (Cu) ions from bioactive silicate (13–93) glass scaffolds on the response of cells in vitro and on bone regeneration and angiogenesis in vivo was studied. Scaffolds doped with varying concentrations of Cu (0–2.0 wt.% CuO) were created with a grid-like microstructure by robotic deposition. When immersed in simulated body fluid in vitro, the Cu-doped scaffolds released Cu ions into the medium in a dose-dependent manner and converted partially to hydroxyapatite. The proliferation and alkaline phosphatase activity of pre-osteoblastic MC3T3-E1 cells cultured on the scaffolds were not affected by 0.4 and 0.8 wt.% CuO in the glass but they were significantly reduced by 2.0 wt.% CuO. The percent new bone that infiltrated the scaffolds implanted for 6 weeks in rat calvarial defects ($46 \pm 8\%$) was not significantly affected by 0.4 or 0.8 wt.% CuO in the glass whereas it was significantly inhibited ($0.8 \pm 0.7\%$) in the scaffolds doped with 2.0 wt.% CuO. The area of new blood vessels in the fibrous tissue that infiltrated the scaffolds increased with CuO content of the glass and was significantly higher for the scaffolds doped with 2.0 wt.% CuO. Loading the scaffolds with bone morphogenetic protein-2 (1 μg/defect) significantly enhanced bone infiltration and reduced fibrous tissue in the scaffolds. These results showed that doping the 13–93 glass scaffolds with up to 0.8 wt.% CuO did not affect their biocompatibility whereas 2.0 wt.% CuO was toxic to cells and detrimental to bone regeneration.

1. Introduction

There is a clinical need for synthetic biomaterials that can reliably repair large (critical size) bone defects. Whereas contained bone defects are repairable with commercially-available, osteoconductive and osteoinductive filler materials [1,2], the repair of large defects in structural bone is a challenging clinical problem. The available treatments such as bone allografts, autografts and porous metals are limited by costs, availability, durability, infection risk, donor site morbidity, and uncertain healing.

The desirable properties of synthetic biomaterials for bone regeneration have been discussed in the literature [3]. Briefly, synthetic biomaterials should be biocompatible, osteoconductive and osteoinductive, and they should have a porous three-dimensional (3D) architecture with interconnected porosity for tissue ingrowth and formation of capillaries [4]. They should also be bioactive or biodegradable, have mechanical properties that match the host bone, have the ability to be formed into anatomically relevant shapes and be sterilizable.

A problem with synthetic biomaterials is that they often lack the osteoinductivity and osteogenicity of autogenous bone grafts, functioning mainly as osteoconductive implants. Consequently, the performance of synthetic biomaterials in vivo is inferior to autogeneous bone grafts (the gold standard for bone repair). The use of synthetic biomaterials by themselves commonly fails to produce clinically significant bone formation in a clinically relevant time [5,6]. In practice, the addition of osteogenic growth factors to synthetic biomaterials is often needed to achieve reliable reconstruction of bone [7,8]. Bone morphogenetic proteins (BMPs) such as BMP2, delivered typically by osteoconductive biomaterials, can induce robust bone formation and they have been used successfully in bone repair [7–9]. However, BMPs are often effective only when used in supra-physiological doses and this has resulted in heightened concerns about adverse biological effects in vivo [10,11].

Angiogenesis is essential for bone formation and growth and plays a critical role in bone defect repair [12]. The relationship between angiogenesis and osteogenesis has been well established [13]. The vasculature transports oxygen, nutrients, soluble factors and numerous cell types to all tissues in the body. The enhancement of angiogenesis should increase osteogenesis [14]. While a variety of approaches have been developed to enhance angiogenesis by synthetic implants, a commonly-

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used approach is the incorporation of angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) [15,16]. However, the use of growth factors can suffer from disadvantages such as high cost, potential adverse biological effects in vivo when used in supra-physiological doses and loss of bioactivity [10,11,17,18].

The release of inorganic angiogenic factors such as metal ions from biomaterials is receiving considerable interest as an alternative approach to growth factors for stimulating angiogenesis and osteogenesis [19–22]. Inorganic ions can have potential advantages, such as low cost, high stability and better clinical safety, when compared to growth factors. In particular, copper (Cu^{2+}) ions have been reported to enhance angiogenesis by stabilizing the expression of hypoxia-inducible factor (HIF-1α), thus mimicking hypoxia, which plays a critical role in the recruitment and differentiation of cells and in blood vessel formation [19,20]. The release of Cu^{2+} ions has been shown to stimulate the expression of proangiogenic factors such as VEGF and transforming growth factor-β (TGF-β) in wounds created in diabetic mice [23,24]. Subcutaneous implantation of Cu-containing borate bioactive glass microfibers in rats significantly enhanced the growth of capillaries and small blood vessels when compared to silicate 45S5 bioactive glass microfibers and sham implant controls [25]. The ionic dissolution product of Cu-doped borate bioactive glass microfibers has been shown to stimulate the expression of angiogenic genes of fibroblasts in vitro and angiogenesis in full-thickness skin wounds in rodents in vivo [26]. Scaffolds of a Cu-doped borosilicate bioactive glass have been reported to enhance blood vessel formation and bone regeneration in rat calvarial defects in vivo [27].

The attractive properties of bioactive glasses as a scaffold material for bone repair have been well described and reviewed in the literature [28,29]. In addition, bioactive glasses can be doped with inorganic ions such as Cu, Zn, Sr and Fe that have been reported to stimulate angiogenesis and/or osteogenesis. As the glass degrades, those ions are released at a therapeutically appropriate concentration. As described above, the release of Cu and other ions from borate and borosilicate bioactive glasses has been reported to enhance angiogenesis in soft tissue repair and both angiogenesis and osteogenesis in osseous defects in vivo. However, borate-based bioactive glasses are not currently approved by the US Food and Drug Administration for in vivo use. Some borate bioactive glasses can also degrade too rapidly for applications in structural bone repair. While silicate bioactive glasses such as 45S5 and 13–93 have been used in bone repair applications for several years [29], the effect of Cu doping on their ability to stimulate angiogenesis and osteogenesis has received little attention.

The objectives of the present study were to create porous scaffolds of silicate 13–93 glass doped with varying concentrations of Cu (0–2.0 wt.% CuO) and evaluate the effect of Cu ions released from the glass on osteoblastic cell response in vitro and on bone regeneration and angiogenesis in vivo. The effects of the Cu ion dopant on bone regeneration were compared with the use of BMP2 addition to the scaffolds (1 μg per scaffold or defect). Synergistic effects on bone regeneration resulting from doping the glass with Cu and loading the scaffolds with BMP2 were also studied in vivo.

2. Materials and methods

2.1. Preparation of scaffolds

Scaffolds of silicate 13–93 bioactive glass (composition $53SiO_2$, $6Na_2O$, $12K_2O$, 5MgO, 20CaO, $4P_2O_5$; wt.%) doped with 0, 0.4, 0.8 and 2.0 wt.% CuO were created with a grid-like microstructure using a robotic deposition (robocasting) method described in detail elsewhere [30]. Briefly, each glass was prepared by melting the requisite amount of reagent grade chemicals (Na_2CO_3, K_2CO_3, $MgCO_3$, $CaCO_3$, SiO_2, $NaH_2PO_4 \cdot 2H_2O$, $Cu(NO_3)_2 \cdot 2.5H_2O$; Fisher Scientific, St. Louis, MO, USA) for 30 min in a platinum crucible at 1350 °C and quenching the molten glass between cold stainless steel plates. The crushed glass was ground in a hardened steel container (8500 Shatterbox®, Spex SamplePrep LLC., Metuchen, NJ, USA) to give particles of size <50 μm. Then the particles were milled for 2 h in an attrition mill (Union Process, Inc., Akron, OH, USA) using water as the liquid medium and ZrO_2 grinding media (3 mm in diameter) to give particles of size 1.0 ± 0.5 μm, as measured by a laser diffraction particle size analyzer (Model LS, Beckman Coulter Inc., CA, USA).

The glass particles were mixed with a 20 wt.% Pluronic-127 binder solution to form a paste (40 vol.% glass particles) and extruded using a robocasting machine (RoboCAD 4.1; 3-D Inks, Stillwater, OK, USA). After drying at room temperature, the scaffolds were heated in O_2 at 0.5 °C/min to 500 °C to burn out the processing additives and sintered for 1 h at 700 °C (heating rate = 5 °C/min) to densify the glass filaments. The as-fabricated scaffolds were sectioned and ground on 320 grit SiC paper to form thin discs (4.6 mm in diameter × 1.5 mm), washed twice with deionized water and twice with anhydrous ethanol, dried in air and sterilized by heating for 12 h at 250 °C.

Thirty-two as-fabricated scaffolds ($n = 8$ per group) were loaded with BMP2 prior to implantation in vivo. The procedure and amount of BMP2 (1 μg per scaffold) were similar to those used previously [31]. Briefly, the scaffolds were reacted for 3 days in an aqueous phosphate solution (0.25 M K_2HPO_4 solution) at 60 °C and a starting pH of 12.0 to convert a thin surface layer (~5 μm) of the glass to HA (or amorphous calcium phosphate, ACP). The mass of the glass scaffolds to the volume of the K_2HPO_4 solution was kept constant at 1 g per 200 ml and the solution was stirred gently each day. The reacted scaffolds were removed from the solution, washed twice with deionized water, and twice with anhydrous ethanol to displace residual water from the scaffolds. Then the scaffolds were dried for at least 24 h at room temperature and stored in a desiccator.

In the BMP2 loading process, a solution of BMP2 (Shenandoah Biotechnology Inc., Warwick, PA, USA) in citric acid was prepared by dissolving 10 μg of BMP2 in 100 μl sterile citric acid (pH = 3.0). Then 10 μl of the BMP2 solution was pipetted on to each scaffold (4.6 mm in diameter × 1.5 mm). The amount of BMP2 loaded into the scaffolds was equivalent to 1 μg per bone defect (or per scaffold) in the animal model. The BMP2 solution was totally incorporated in the converted surface layer of the scaffolds and there was no visible evidence for any of the solution flowing out of the scaffolds. After loading with BMP2, the scaffolds were kept for ~24 h in a refrigerator at 4 °C to dry them prior to implantation.

2.2. Degradation and conversion of scaffolds in simulated body fluid (SBF) in vitro

The degradation of the bioactive glass scaffolds (4.6 mm in diameter × 1.5 mm) and their conversion to HA were studied as a function of immersion time in SBF [32] with a starting pH of 7.4. The weight loss of the scaffolds was measured as a function of time and used as a measure of the conversion of the glass to HA while the pH of the medium was measured using a pH meter, as described previously [33,34]. A ratio of 1 g of scaffold to 100 ml of SBF was used in all of the conversion experiments. The concentration of Cu ions released from the scaffolds into the SBF during the degradation and conversion process was determined using inductively-coupled plasma optical emission spectrometry, ICP-OES (Optima 2000DV; Perkin Elmer, Waltham, MA). The weight loss, pH and Cu concentration at each time point were measured in triplicate and the data were expressed as a mean ± standard deviation (SD).

The formation of HA on the surface of the scaffolds was characterized using scanning electron microscopy, SEM (S-4700; Hitachi, Tokyo, Japan) and Raman spectroscopy (Horiba-Jobin Yvon, Inc., Edison, NJ). The samples were coated with Au/Pd and examined in the SEM at an accelerating voltage of 15 kV and a working distance of 12 mm. Energy-dispersive X-ray (EDS) analysis in the SEM was used to determine the