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The proangiogenic potential of a novel calcium releasing biomaterial: Impact on cell recruitment

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ABSTRACT

In current bone tissue engineering strategies the achievement of sufficient angiogenesis during tissue regeneration is still a major limitation in order to attain full functionality. Several strategies have been described to tackle this problem, mainly by the use of angiogenic factors or endothelial progenitor cells. However, when facing a clinical scenario these approaches are inherently complex and present a high cost. As such, more cost effective alternatives are awaited. Here, we demonstrate the potential of electrospun poly(lactic acid) (PLA) fiber-based membranes, containing calcium phosphate ormoglass (CaP) particles, to elicit angiogenesis *in vivo*, in a subcutaneous model in mice. We show that the current approach elicited the local expression of angiogenic factors, associated to a chemotactic effect on macrophages, and sustained angiogenesis into the biomaterial. As both PLA and CaP are currently accepted for clinical application these off-the-shelf novel membranes have great potential for guided bone regeneration applications.

Statement of significance

In current bone tissue engineering approaches the achievement of sufficient angiogenesis, during tissue regeneration, is a major limitation in order to attain full tissue functionality. Recently, our group has found that calcium ions released by the degradation of calcium phosphate ormoglasses (CaP) are effective angiogenic promoters. Based on this, in this work we successfully produced hybrid fibrous mats with different contents of CaP nanoparticles and thus with different calcium ion release rates, using an ormoglass – poly(lactic acid) blend approach. We show that these matrices, upon implantation in a subcutaneous site, could elicit the local expression of angiogenic factors, associated to a chemotactic effect on macrophages, and sustained angiogenesis into the biomaterial, in a CaP dose dependent manner. This off-the-shelf cost effective approach presents great potential to translate to the clinics.

1. Introduction

Currently, bone regeneration strategies still face major limitations, exacerbated by the increased incidence of bone pathologies linked to the general population aging profile. Present regeneration approaches focus on the following main strategies: (A) the use of autologous bone grafts, having as main limitations the insufficient availability of healthy bone tissue at the donor site, the complexity of the surgery and associated morbidity [1,2]; (B) allogeneic bone grafts, associated with immunogenic and infectious risks [3]; and (C) the use of bone substitutes, that in spite of their great potential to satisfy clinical need, have shown insufficient vascularization and osteointegration, when in the absence of autologous cells [4]. Indeed, the insufficient vascularization is considered as the major limitation in order to attain construct integration and tissue functionality. This drawback is further relevant when considering large bone defects, where the amount of oxygen required for cell survival is limited to a diffusion distance between 150–200 μm from

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the supplying blood vessels [5]. Several strategies have been described to tackle this problem, mainly by the use of angiogenic factors or endothelial progenitor cells [6]. However, when facing a clinical scenario these approaches are inherently complex and present a high cost.

In the context of bone tissue engineering, it has been shown that angiogenesis and osteogenesis are closely linked, where both processes have to be tightly regulated in order to achieve successful bone tissue regeneration [6–9]. As such, in view of the development of a biomaterial for bone repair, both angiogenesis and osteogenesis have to be taken into account. Additionally, in view of a broad clinical application, the establishment of off-the-shelf strategies may prove beneficial and reduce regulatory issues. Hence, the use of synthetic polymeric matrices, that can be readily engineered and custom designed, may show the future for new bone therapeutic approaches. Such an example is the synthetic polymer poly(lactic acid) (PLA), being this biocompatible and degradable polyester been proposed for a wide range of bone regeneration approaches. Nonetheless, it possesses poor osteoinductive and angiogenic properties. The association with inorganic components, in order to form a composite material, can then offer the potential to improve material bioactivity, while maintaining mechanical properties. In this view, the combination of inorganic moieties with the capacity to release, in a controlled fashion, bioactive ions is a simple and cost effective approach that can show alternatives in order to achieve ready-to-use bone substitutes. We have recently demonstrated that the increased local concentration of calcium (Ca) ions could induce the migration, maturation and organization of endothelial progenitors [10].

Following this rational, here we explored the formation of a composite membrane, based on electrospun PLA containing Ca phosphate (CaP) ormoglass (organic modified glass) nanoparticles (NP), in order to achieve improved bone angiogenesis. Indeed, in another approach and using different material conformation (i.e. solvent casting) we could show that this composite could elicit proangiogenic and chemotactic properties on endothelial precursor cells, *in vitro* [11].

As such, we developed different formulations of PLA-electrospun composite membranes, containing different percentages of CaP ormoglass NPs and with different calcium release profiles and tested their subsequent impact on angiogenesis, using a pre-clinical subcutaneous model *in vivo*. Increased extracellular calcium levels have shown to promote the chemotaxis of a wide range of cell types [12], including monocytes [13] and endothelial cells [10]. As such, we evaluated the proangiogenic output of these membranes *in vivo* and assessed their impact on the local expression of proangiogenic factors and on cell recruitment.

2. Materials and methods

2.1. Reagents

Unless mentioned otherwise, all reagents were obtained from Sigma–Aldrich and were of analytical grade.

2.2. Membrane production and characterization

CaP ormoglass NPs were prepared by a partial hydrolyzed alkoxides sol–gel method with a composition of 44.5:44.5:6:5 $CaO:P_2O_5:Na_2O:TiO_2$ molar ratio, following a previous report [14]. Ca and Na precursor solutions were prepared by refluxing metallic Ca and Na in 2-methoxyethanol. P precursor solution was obtained by refluxing P_2O_5 in absolute ethanol. Ti tetraisopropoxide was commercially obtained (ALFA AESAR, 97%) and diluted in absolute ethanol. After mixing Ca, Na an Ti precursor solutions in the proper molar ratio in inert atmosphere, a catalyst with a composition related to Ti ($Ti:H_2O:NH_3$:EtOH 1:60:0.3:12) was added at 4 °C at 2 ml/h using an infusion pump. Afterwards, P precursor was similarly added at 4 °C and 1 ml/h flow. The mix turned from dark brown to clear orange and it was subsequently hold at 70 °C during 4 days for aging and allow the formation of NPs. Nanoparticles were collected and washed with absolute ethanol by centrifugation and dried at 90 °C in the oven.

10%, 20% and 30% w/w loaded PLA nanofibers (NP10, NP20 and NP30) were prepared by ultrasonic dispersion of the nanoparticles in a 4% PLA (Purasorb PLDL 7038, inherent viscosity midpoint 3.8 dl/g, molecular mass ≈850,000 Da) solution. Then, the fibers were conventionally electrospun at 8 kV, 15 cm distance tip-collector and at 1200 rpm rotary collector speed.

2.3. Calcium release and FE-SEM characterization

Calcium release measurement was performed by preparing three square samples of 1.7 cm × 1.7 cm × 55 μm for each material (PLA, PLA NP 10, NP 20 and NP 30). They were separately introduced in a 24 well plate and fixed with Teflon rings. 500 μl of culture medium, advanced DMEM containing 15% (v/v) FBS, 1% (v/v) L-Glutamine and 1% (v/v) Penicilin/Streptomycin) was added to each well. The samples were incubated at 37 °C and the medium was replaced at different time points during two days. The calcium concentration of each replaced medium was measured by spectrophotometry using a chromophore (O-Cresolphthalein Complexone).

Micro- and Nano-morphology was measured using an Ultra-High Resolution Field Emission Scanning Electron Microscopy (Nova Nano SEM-230; FEI Co., Netherlands), operating at 5.00 kV and coating the samples with an ultra-thin layer of carbon to make them conductive.

2.4. Cell culture

Human progenitor-derived endothelial cells (PDECs) were obtained as previously described [15]. Briefly, human umbilical cord blood from healthy donors (between 20 and 35 years) were diluted with one part of PBS, 2% (v/v) fetal calf serum (FCS) and 2 mM ethylene diamine tetraacetic acid (EDTA), and applied to a density gradient centrifugation in Histopaque® solution (1.077 g ml^{-1}). Mononuclear cells were recovered from the buffy coat, washed several times with PBS and cultured in endothelial cell growth medium-2 (EGM-2; Lonza-Verviers, France) containing all kit supplements and 5% (v/v) FCS (GIBCO Life Technologies, Karlsruhe, Germany), on collagen-coated 12-well plates (collagen type I from rat tail, BD Biosciences) and at 5×10^7 cells per cm^2. At day 4, non-adherent cells were removed and medium was refreshed every other days. After 2–3 weeks, cobblestone-like morphology colonies were harvested, using 0.25% (w/v) trypsin–EDTA (GIBCO) and subcultured in fresh collagen-coated dishes. Cells were expanded over several passages, using standard cell culture procedures.

2.5. In vitro evaluation

Before cell seeding, electrospun scaffolds (24 mm in diameter) were submitted to UV radiation for 30 min for sterilization. PDECs were seeded over the materials at $20 \times 10^3/cm^2$, for 1, 3 and 7 days in endothelial cell growth medium-2 (EGM-2; Lonza-Verviers, France).

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