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Adsorbed fibrinogen leads to improved bone regeneration and correlates with differences in the systemic immune response

S.G. Santos [a,*,1], M. Lamghari [a,b,1], C.R. Almeida [a], M.I. Oliveira [a], N. Neves [a,c,d], A.C. Ribeiro [a], J.N. Barbosa [a,b], R. Barros [a,e], J. Maciel [a,e], M.C.L. Martins [a,b], R.M. Gonçalves [a], M.A. Barbosa [a,b]

[a] Instituto de Engenharia Biomédica, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal
[b] Instituto Ciências Biomédicas Abel Salazar da Universidade do Porto, Rua de Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal
[c] Spine Group, Orthopedic Department, Hospital de São João, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal
[d] Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200 Porto, Portugal
[e] Faculty of Engineering, University of Porto, R. Dr. Roberto Frias, s/n 4200-465 Porto, Portugal

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ABSTRACT

Designing new biomaterials that can modulate the inflammatory response instead of attempting just to reduce it constitutes a paradigm change in regenerative medicine. This work aimed to investigate the capacity of an immunomodulatory biomaterial to enhance bone regeneration. For that purpose we incorporated a molecule with well-established pro-inflammatory and pro-healing roles, fibrinogen, in chitosan scaffolds. Two different incorporation strategies were tested, leading to concentrations of 0.54 ± 0.10 mg fibrinogen g^{-1} scaffold immediately upon adsorption (Fg-Sol), and 0.34 ± 0.04 mg fibrinogen g^{-1} scaffold after washing (Fg-Ads). These materials were implanted in a critical size bone defect in rats. At two months post-implantation the extent of bone regeneration was examined by histology and the systemic immune response triggered was evaluated by determining the percentages of myeloid cells, T and B lymphocytes in the draining lymph nodes. The results obtained indicate that the fibrinogen incorporation strategy conditioned the osteogenic capacity of biomaterials. Fg-Ads scaffolds led to more bone formation, and the presence of Fg stimulated angiogenesis. Furthermore, animals implanted with Fg-Ads scaffolds showed significant increases in the percentages of B lymphocytes and myeloid cells in the draining lymph nodes, while levels of T lymphocytes were not significantly different. Finally, a significant increase in TGF-β1 was detected in the plasma of animals implanted with Fg-Ads. Taken together the results presented suggest a potential correlation between the elicited immune response and biomaterial osteogenic performance.

1. Introduction

It is well established that implanted materials always elicit an inflammatory reaction, leading often to a foreign body reaction [1]. For many years, inflammation has been considered an adverse reaction to implant biomaterials, an idea dictated by failure of implanted devices, often non-degradable, as a result of severe inflammation. It has been only in recent years that the paradigm of "fighting inflammation" has been gradually replaced by the concept of "modulating inflammation" [2]. Although some biomaterials scientists have advocated this shift, the fact is that the concept of immuno-modulatory biomaterials remains largely unexplored. Moreover, the role of immune cells and inflammatory mediators in biomaterial-mediated bone repair/regeneration has been considerably overlooked. It is not uncommon to find studies reporting the absence of (or mild) inflammatory reactions, associated with degradable biomaterials developed for tissue repair/regeneration [3,4].

The inflammatory response to implantable devices is greatly influenced by the material surface chemistry, as demonstrated previously by others [5] and us [6–9]. Using self-assembled monolayers (SAMs) we demonstrated that methyl-terminated SAMs cause early inflammatory cell recruitment in vivo [8], more polymorphonuclear leukocytes (PMNs) than mononuclear cells [9] and the formation of thicker fibrous capsules around implanted materials [6]. Additionally, biomaterials surface chemistry is crucial in controlling the inflammatory response to biodegradable polymers, as shown for chitosan (Ch) scaffolds, for which a low degree of acetylation (DA = 4%) induces milder inflammation than higher DA (DA = 15%) [10].

Nowadays, the use of hybrid biomaterials, composed of a material that is combined with a bioactive molecule (e.g. antiresorptive

* Corresponding author. Tel.: +351 226074900; fax: +351 226094567.
E-mail address: susana.santos@ineb.up.pt (S.G. Santos).
[1] These authors contributed equally to this work.

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or osteogenic), is one of the central strategies being explored for bone repair [3,4,11]. However, promotion of bone regeneration through inflammatory signals remains largely unexplored, in spite of evidence that inflammation may have a positive role in bone remodeling. For instance, mice deficient in TNF-α show delayed endochondral bone formation by several weeks, indicating that TNF-α production is advantageous in the early stages of bone repair [12]. However, sustained TNF-α production impairs bone formation [13]. These apparently contradictory data support the idea that the effect of TNF-α is time- or dose-dependent [12]. Also, osteolysis is modulated by inflammation, since differentiation of monocyte/macrophage lineage precursors into osteoclasts is controlled by macrophage colony-stimulating factor (M-CSF) [14] and by receptor of NF-KB ligand (RANKL), which can be produced by osteoblasts, activated T lymphocytes and NK cells [15].

In this study we have used a fibrinogen-coated biomaterial to explore the role of inflammation in bone regeneration. Fibrinogen (Fg) interaction with platelets and leukocytes has been previously studied in our group [16]. The rational for using this pro-inflammatory protein to stimulate osteogenesis was based on the hypothesis that Fg, when adsorbed to an adequate substrate, might trigger a balance between pro- and anti-inflammatory events. Importantly, modification of Fg by covalently linking it to PEG–DA (poly(ethylene) glycol–diacrilate) has been shown to result in a series of hydrogels with varying degrees of osteogenic properties [17]. It is also known that the dose of Fg is critical for wound healing in mice [18]. Furthermore, in vitro assays demonstrated that Fg adsorption to two-dimensional (2-D) surfaces alters NK cell behavior and leads to an increase in NK cell-mediated mesenchymal stromal/stem cell (MSC) recruitment [19].

As a substrate for Fg immobilization, the natural polysaccharide Ch has been selected. Ch is biodegradable and is obtained by N-deacetylation of chitin. This biomaterial has a well-established biocompatibility and has been investigated for several biomedical applications, such as wound dressings [20], drug and gene delivery [21,22] and bone tissue engineering [23]. Ch is a linear copolymer of glucosamine and N-acetyl glucosamine in a beta1-4 linkage. The degree of N-acetylation (DA), which represents the molar fraction of N-acetylated units, is an important parameter used to characterize Ch. The DA influences solubility, charge density and susceptibility to degradation. Higher DAs lead to faster biodegradation rates [24,25]. Ch influences the polarization of macrophages, inducing the production of cytokines and the expression of activation markers characteristic of an M2c subset [26]. Upon implantation, Ch has been reported to lead to an inflammatory reaction that is less pronounced for lower DA [10,27] of the polymer, and has shown a high degree of biocompatibility in different conditions [27–30].

In this study we investigated in vivo the different biological responses to a combination biomaterial of Ch with Fg. Of note, two combinations were tested which differed in the amount and form of incorporation of the Fg molecule. The latter was achieved by delivering Fg in soluble or adsorbed forms, using Ch scaffolds as a delivery system. The scaffolds were implanted in a load-bearing critical size bone defect in the rat femur. After 2 months of implantation the histological evaluation of bone regeneration was combined with an analysis of the immune cell populations present in the draining lymph nodes, where an adaptive immune response could be more readily detectable.

2. Materials and methods

2.1. Preparation of Ch three-dimensional (3-D) scaffolds

Ch 3-D scaffolds were prepared by freeze-drying. Firstly, Ch (France-Chitine) was purified as described previously [31]. Briefly, it was dried, hydrated and dissolved for 1 h in HCl at 0.1 M (Merck). The solution was filtered through 100, 41 and 20 μm pore size filters (Millipore) with a vacuum pump. After filtration, Ch was precipitated with 0.1 M KOH (Merck). Ch was washed with MilliQ water until reaching a neutral pH and was placed in the freezer overnight at −80 °C. Ch was then freeze-dried (Freezone 2.5, Labconco) and finally milled (IKA mill) to obtain a fine powder.

Scaffolds were produced as previously described [32]. Briefly, a 2% solution of purified Ch (degree of acetylation (DA): 12.00 ± 2.35%, molecular weight (MW): $324 \pm 27 \times 10^3$) [31] was hydrated overnight at 4 °C and dissolved by adding acetic acid (Panreac) to a final concentration of 0.2 M under strong vortex agitation. The Ch solution was incubated for 24 h at 4 °C, was centrifuged at 4165 g for 5 min and then 800 μl was added to each well of a 48-well plate. Plates were placed at −20 °C and freeze-dried at −80 °C for 48 h to produce scaffolds. The latter were removed from the plate and cut in the shape of cylinders with a diameter of 4 mm and a height of 5 mm (2.3 mg average weight). For disinfection, scaffolds were impregnated under vacuum in a gradient of ethanol solutions (99.9% for 10 min, 70% for 30 min, 50% and 25% for 10 min each), followed by three 10 min washes in sterile phosphate buffered saline (PBS). Scaffolds were maintained in sterile PBS at 4 °C protected from light overnight.

2.2. Scanning electron microscopy characterization of Ch 3-D scaffolds

Cross-sections of 1 mm thickness were cut and mounted with carbon tape, for scanning electron microscopy (SEM) analysis. Samples were sputter-coated with gold and observed with a JEOL JSM-6301F SEM, at 15 kV and amplifications of 30× or 250×. Pore diameter and interconnecting pore diameter were measured in four different scaffolds.

2.3. Measurement of endotoxin levels

Ch endotoxin levels were measured in Ch water extracts and in Fg dissolved in endotoxin-free water. Ch extracts were prepared using 40 ml endotoxin-free water g^{-1} Ch, incubating the Ch suspension for 24 h at 50 °C under continuous shaking (250 rpm), as described elsewhere [33].

Endotoxin levels were measured using the Limulus Amebocyte Lysate (LAL) QCL-1000 test (Lonza), for the chromogenic quantitation of Gram-negative bacterial endotoxin, according to the Food and Drug Administration guidelines on the validation and use of the LAL test as an end-product endotoxin test for human and animal parental drugs, biological products and medical devices. Ch extracts and Fg solution revealed endotoxin levels lower than 0.1 EU ml^{-1} (EU: unit of measurement for endotoxin activity), which are far below the recommended FDA limit (0.5 EU ml^{-1}).

2.4. Incorporation of human fibrinogen (Fg)

Fg (Sigma, F4129) (0.1 mg ml^{-1}) was incorporated in Ch scaffolds under sterile conditions using two different strategies. In the first method Ch scaffolds were immersed in a Fg solution (0.1 mg ml^{-1} in PBS), six scaffolds in 5 ml, during 2 h under vacuum, to ensure that open pores were totally filled with the Fg solution. After this period, the scaffolds, designated as Fg-Sol, were immediately used for implantation. In the second method, Ch scaffolds were immersed in PBS in a falcon tube and subjected to vacuum during 1 h, to remove air bubbles inside the pores. After 1 h, the scaffolds were individually transferred to another falcon using tweezers and Fg (0.1 mg ml^{-1} in PBS) was adsorbed to Ch scaffolds during 2 h under vacuum, as before. After this period, the scaffolds were transferred to another tube and washed twice in PBS, the scaffolds were squeezed gently before each wash. After this wash-

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