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Fibrinogen scaffolds with immunomodulatory properties promote *in vivo* bone regeneration

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ABSTRACT

The hypothesis behind this work is that fibrinogen (Fg), classically considered a pro-inflammatory protein, can promote bone repair/regeneration. Injury and biomaterial implantation naturally lead to an inflammatory response, which should be under control, but not necessarily minimized. Herein, porous scaffolds entirely constituted of Fg (Fg-3D) were implanted in a femoral rat bone defect and investigated at two important time points, addressing the bone regenerative process and the local and systemic immune responses, both crucial to elucidate the mechanisms of tissue remodelling. Fg-3D led to early infiltration of granulation tissue (6 days post-implantation), followed by bone defect closure, including periosteum repair (8 weeks post-injury). In the acute inflammatory phase (6 days) local gene expression analysis revealed significant increases of pro-inflammatory cytokines IL-6 and IL-8, when compared with non-operated animals. This correlated with modified proportions of systemic immune cell populations, namely increased T cells and decreased B, NK and NKT lymphocytes and myeloid cell, including the Mac-1+ (CD18+/CD11b+) subpopulation. At 8 weeks, Fg-3D led to decreased plasma levels of IL-1β and increased TGF-β1. Thus, our data supports the hypothesis, establishing a link between bone repair induced by Fg-3D and the immune response. In this sense, Fg-3D scaffolds may be considered immunomodulatory biomaterials.

1. Introduction

Upon injury and/or biomaterial implantation there is an inflammatory response, which is required for the regenerative process to begin. This inflammatory response to implantable, and particularly non-degradable biomaterials, can culminate in a foreign body reaction, which is related with the failure to establish a pro-regenerative environment [1]. First generations of materials applied in clinics aimed at restoration of the physical properties of the damaged tissues, while minimizing or even avoiding the immune response. Most of the currently used dental and orthopaedic

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implants are examples of biomaterials that still follow that strategy [2]. However, in terms of biomedical research the paradigm is shifting from “fighting inflammation” to “modulating inflammation” [3].

Inflammation, repair and remodelling are the three stages that compose bone healing [4]. First, a blood clot forms after bone injury, which provides a temporary matrix for immune cell recruitment to the injury site. Polymorphonuclear leukocytes (PMN) quickly migrate to bone injury and interact with damaged tissue during the first 24 h. Monocytes/Macrophages and lymphocytes (NK, T and B cells) are attracted to the injury in the next days [5]. Although acute inflammation is described as lasting 4 days and the chronic inflammation to be over in about 2 weeks, immune cells have an active role throughout bone repair and remodelling [4,5]. In fact, the balance between cytokines, chemokines and immune cell populations in the injury microenvironment is essential for tissue regeneration. This can be impaired by infection or chronic inflammatory conditions, such as autoimmune diseases and the foreign body response against a biomaterial, and when inflammation does not resolve and tissue healing is impaired [4,6]. In agreement, previous work has shown that successful osteointegration of implants correlates with systemic changes in immune cells [7].

Fibrinogen (Fg) is a blood protein involved in blood clotting. During haemorrhage, a fibrin clot is formed from Fg cleaved by thrombin, which prevents extensive blood loss. The Fg-derived clot is the primordial extracellular matrix (ECM) that supports tissue regeneration, thus providing Fg with pro-healing properties [8,9]. Two arginine-glycine-aspartate (RGD) motifs were identified in Fg structure [10], which are related with improved cell adhesion to Fg-modified materials [11]. Additionally, Mac-1, an important receptor of activated immune cells, finds numerous binding sites on the Fg molecule [12]. Also, vascular endothelial growth factor (VEGF), an important factor for neovascularization, binds Fg with high affinity [13], what may explain the improved angiogenesis induced by Fg [7,14].

Clinically, fibrinogen is applied together with thrombin as fibrin hydrogels that are used as biological adhesives [15]. Beyond their well-known haemostatic and sealant properties, alternative applications in tissue engineering have been tested, combining fibrin with ceramics, cells or other proteins [16–20]. Although the use of thrombin is a standard in fibrin sealants, it increases the risk of thrombosis and life-threatening complications.

The strategy of Fg delivery appears to be of paramount importance to the host response. Soluble Fg does not enhance wound healing [11], instead elicits autoimmunity and nervous tissue damage [21]. The pro-regenerative potential of Fg-incorporating biomaterials has been previously reported by us [7] and others [22]. We have explored the potential of adsorbed Fg to modulate immune cell responses and induce regeneration. Materials modified with Fg led to increased recruitment of mesenchymal stem/stromal cells (MSC) mediated by different immune cells [23], downregulation of pro-inflammatory molecules and up-regulation of bone and angiogenic factors secreted by macrophages [24]. The degradation of chitosan films by osteoclasts was also accelerated by Fg adsorption [25]. Most importantly, our previous work showed that implantation of Fg-modified chitosan scaffolds in a femoral critical bone defect led to increased angiogenesis and new bone formation at the defect periphery, together with significant changes in myeloid and B cell populations in the draining lymph nodes [7].

Due to the potential revealed by Fg-modified materials [7,23,24], whole-Fg scaffolds (Fg-3D) were here produced to assess the hypothesis that, when stabilized in a 3D porous structure, Fg can promote a pro-regenerative microenvironment, mimicking the blood clot. Fg-3D scaffolds were produced by freeze drying, without addition of any exogenous enzymatic compound, and their capacity to stimulate bone repair was addressed. For that, Fg-3D were extensively characterized by SEM, ATR-FTIR and NMR, their degradation profile, cytotoxicity and endotoxin levels were assessed, following the international standard ISO 10993-5:2009. Fg-3D scaffolds were then implanted in a load-bearing bone defect in the rat femur. Local and systemic immune responses were analysed at two critical time points post-implantation, 6 days and 8 weeks. A combination of flow cytometry and ELISA was used to investigate the systemic response, while qRT-PCR complemented the histological analysis of the local response. Bone repair was also more closely evaluated by micro-CT. By assessing the early and long-term biological response we aim at understanding the impact of Fg-3D on the inflammatory response and subsequent influence on bone tissue repair (Fig. 1).

2. Materials and methods

2.1. Preparation of Fg-3D scaffolds

Fibrinogen 3D scaffolds (Fg-3D) were prepared by freeze-drying, similarly to chitosan scaffolds previously prepared by our group [7]. A solution of human Fg (fraction I, type III from human plasma; cat. F4129, Sigma), 70 mg/mL, was prepared in Phosphate Buffered Saline Solution (PBS) at neutral pH (7.4). The solution was then casted into 48-well plate (800 μL/well), frozen overnight at −20 °C in a horizontal surface and freeze-dried at −80 °C for 48 h to produce scaffolds. These were removed from the plate and cut in the shape of cylinders with 4 mm diameter and 5 mm height. Scaffolds were neutralized and disinfected through impregnation 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 PBS. Fg-3D scaffolds were maintained overnight in sterile PBS at 4 °C protected from light, before further analysis or implantation.

2.2. Scanning electron microscopy characterization

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 magnifications of 30× or 250×. Twenty-five pores and interconnecting pores were measured in a representative scaffold to determine the range of pore sizes.

2.3. ATR-FTIR spectroscopy

Previously to FTIR analysis, all samples were dried in a vacuum chamber overnight at room temperature. ATR-FTIR spectra of Fg powder, lyophilized Fg scaffolds and after ethanol neutralization (Fg-3D) were obtained using a FTIR spectrophotometer (SpectrumTwo, Perkin Elmer). All samples were submitted to the same pressure and 16 scans were collected with 4 cm^{-1} resolution. Peak analysis was performed through spectra analysis and evaluation of first and second derivatives.

2.4. NMR analysis

The ^{13}C NMR spectra were recorded using a Bruker Avance III (9.4 T) spectrometer operating at 400 MHz for proton and a 4-mm double-bearing magic-angle spinning (MAS) probe. For the ^{13}C cross-polarization and MAS (CP-MAS) NMR experiments, we used 90° pulse lengths of 3–5 μs, a 2 ms contact time, a 5 s recycle delay and a spinning rate of 12 kHz. For the ^{13}C Single Pulse Excitation