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Fibrinogen promotes resorption of chitosan by human osteoclasts

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ABSTRACT

The osteoconductive and osteoinductive properties of materials intended for bone regeneration have been extensively tested, but the resorbability of these materials is often overlooked. Osteoclasts are responsible for bone resorption and play a crucial role in bone remodeling, which is essential for complete regeneration of bone tissue following injury. In this study we compare, for the first time, the ability of unmodified and fibrinogen (Fg)-modified chitosan (Ch) substrates to support the formation of multinucleated osteoclasts, and the potential of these cells to resorb the two substrates in vitro. Osteoclasts were differentiated from primary human peripheral blood monocytes directly on the substrates being investigated. Our results showed similar cell adhesion to unmodified and Fg-modified Ch substrates. Although the number of multinucleated osteoclasts on both Ch substrates increased throughout the culture period, by 21 days of culture significantly more highly multinucleated osteoclasts (>10 nuclei per cell) were observed on Fg-modified Ch, when compared to Ch alone. In addition, cells were tartrate-resistant acid phosphatase positive and secreted significantly more enzyme on Ch-based substrates than in control conditions. Unmodified and Fg-modified Ch resorption was investigated by fluorescence microscopy and confirmed by electron microscopy. Quantification of results obtained by fluorescence microscopy shows that Fg modification led to significantly higher substrate resorption by 17 days of culture. Our results show that osteoclasts, beyond resorbing mineralized substrates, successfully resorb a polymeric substrate (Ch), with Fg accelerating this process. Thus, in bone tissue regeneration strategies employing polymeric biomaterials, resorption may depend not only on macrophages, but also on osteoclasts.

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1. Introduction

Conventional biomaterials have a limited ability to modulate the repair and regeneration of bony host tissues, which has impaired their use for tissue engineering applications. To overcome this limitation, various strategies have been adopted and new materials are currently being developed [1]. In particular, efforts are being made to control cell-binding interactions, release appropriate growth factors or provide environmental cues to condition cellular responses [2].

In the case of materials for bone regeneration, biomaterial degradation/resorption at a controlled rate and by the cells involved in bone remodeling has also been pursued [3], but to a lesser extent. However, if effective osseointegration and bone regeneration are to be achieved, scaffolds that can support self-healing processes, by permitting a coordinated regulation of osteoclasts and osteoblasts, have to be designed. Osteoblasts and their precursor cells have attracted a lot of interest, particularly regarding their attachment, spreading and proliferation in response to a given biomaterial [4–7]. Conversely, the capacity of a specific substrate to induce and support osteoclastogenesis, or the ability of osteoclasts to resorb different biomaterials, remains largely to be elucidated. Nonetheless, cell-based assays replicate more realistic biological conditions than acellular environments, such as in simulated body fluid degradation assays, and osteoclasts are the most widely used cells for this purpose [8].

Osteoclasts derive from pluripotent hematopoietic stem cells [9] and are responsible for resorbing bone, as part of the process of bone remodeling [10–12]. During physiological bone resorption systemic hormones influence bone stromal cells, especially osteoblasts [13], stimulating the production of the receptor activator of nuclear factor-k ligand (RANKL), which is essential for osteoclast formation and function [14] and macrophage-colony stimulating factor (M-CSF), also a critical factor for generating osteoclasts, both in vivo and in vitro [15]. Both cytokines are known to promote active osteoclast survival and induce their differentiation, even in the absence of osteoblastic cells [16,17]. As differentiation proceeds, osteoclasts merge, originating large multinucleated osteoclasts with increased substrate resorption ability [18]. To initiate bone

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resorption, osteoclasts adhere to the substrate, forming a sealing zone, an actin ring that closes the bone matrix in roughly circular areas and prevents the leakage of acids and protons produced during bone resorption [19]. Throughout that process, osteoclasts also secrete enzymes onto the bone to digest collagen fibers and other matrix proteins, including cathepsin K, which is essential for bone resorption and tartrate-resistant acid phosphatase (TRAP), whose secretion is normally correlated with resorptive behavior [18,20,21]. Degraded bone fragments are endocytosed and transported to the basolateral side of osteoclasts [22,23]. Following osteoclast-mediated bone resorption, mature osteoblasts secrete bone matrix, which becomes mineralized [24]. Thus, the ability of osteoclasts to colonize and resorb a biomaterial for bone implantation could promote better osseointegration and even bone regeneration via a process similar to bone remodeling.

Chitosan (Ch) is a chitin-derived natural polymer, which has gained popularity due to its good biocompatibility, biodegradability and ease of processing [25–29]. Ch has been shown to be suitable for use in scaffolds for bone regeneration strategies, as it can be employed to produce structures with appropriate porosity and can support osteoblastic cell attachment and proliferation [30]. When used as a thin coating on microstructured titanium surfaces Ch induces release of osteocalcin and osteoprotegerin [7]. In addition, Ch is able to bind to extracellular matrix (ECM) components, growth factors and cytokines, concentrating added mediators near to the growth site [31]. Interestingly, a previous report has shown that osteoclasts can attach to Ch and that Ch modification with collagen promotes osteoclastogenesis [32]. It is noteworthy that Ch can be degraded in vivo [33,34], and hydrolases such as lysozymes, cellulases, chitosanases and chitinases, among others, have been shown to be involved in that process. As an example, chitotriosidase is a chitinase expressed in human leukocytes [35] that recognizes chitin and Ch, and generates small diffusible chitin/Ch fragments, capable of activating human macrophages [36,37]. Although products resulting from the degradation of synthetic polymers are known to be inflammatory, potentially damaging the surrounding tissue [38], more recent reports revealed that Ch shows anti-inflammatory properties [39] and that its degradation products can support angiogenesis [40].

In this study, Ch substrates were left unmodified or modified by adsorption of human Fg, a protein present in blood plasma. Fg is essential for the coagulation of blood, and is required for normal platelet function and wound healing [41]. Importantly, it has been proposed that Fg plays a significant role in tissue repair by stabilizing wound fields and supporting local cell proliferation and migration of inflammatory, endothelial and stromal cells [42]. Moreover, a recent report from our laboratory has shown that Fg adsorption to Ch can modulate NK cell behavior, including their capacity to recruit mesenchymal stem/stromal cells [43]. Finally, a previous study reports that poly(ethylene glycol) conjugated to Fg materials have potential osteogenic properties in vivo, which the authors correlate with an appropriate degradation rate [44].

In this context, we hypothesized that modifying Ch with the pro-inflammatory molecule Fg would increase the capacity of Ch to support the formation of osteoclasts. Moreover, we addressed the ability of this cell population to resorb Ch. Our results indicate that Ch and Fg-modified Ch support osteoclastogenesis and that Fg promotes formation of larger, more multinucleated osteoclasts. Resorption of Ch substrates is also enhanced upon Fg adsorption.

2. Materials and methods

2.1. Ch purification

High molecular weight (Mw) Ch (France Chitine, Orange, France, degree of *N*-acetylation 11–12%, Mw $324 \pm 27 \times 10^3$, endotoxin-free) was purified as described [45]. Briefly, Ch was hydrated using Milli-Q water, and then dissolved in 0.1 M HCl under stirring and filtered. Ch was next precipitated with 0.1 M KOH, washed with Milli-Q water, freeze-dried (48 h) and finally milled (IKA, Germany) until a fine powder was obtained.

2.2. Preparation of Ch films

After purification, Ch was dissolved in 0.2 M acetic acid (AcOH) to a final concentration of 0.5% and the solution was degassed, filtered through 0.2 μm pore filters (Millipore, MA, USA) and stored at 4 °C, protected from light until use. Ch films were prepared using a method adapted from one previously described by us [4]. Briefly, a drop of Ch solution was deposited directly into each well of a 96 well (80 μl per well) or a 24 well (200 μl per well) tissue culture polystyrene (TCPS) plate (BD, Biosciences, NJ, USA) and allowed to dry at 37 °C for 48 h. Films were then neutralized with 0.1 M NaOH, washed twice with distilled water and dried overnight at 37 °C. Prior to cell culture, films were disinfected with filtered 70% ethanol and washed twice with phosphate-buffer saline (PBS). Where indicated, Ch films were modified with Fg (Sigma–Aldrich), by incubation for 2 h with a 100 $\mu g\ ml^{-1}$ Fg solution in PBS. Before cell culture all films were equilibrated with complete culture medium for 1 h at 37 °C.

2.3. Preparation of FITC-labeled Ch films

To prepare fluorescent Ch, a 5% Ch modification—in which 5% of amine groups were labeled with fluorescein isothiocyanate (FITC) (Sigma–Aldrich)—was performed. Purified Ch was dried overnight, in a vacuum oven, and 400 mg were then hydrated in 400 ml of 1% (v/v) AcOH at 4 °C until complete dissolution. Then, 44 mg of FITC were dissolved in 400 ml of methanol and mixed with 400 ml of Ch solution to achieve an estimated 5% modification, as calculated and performed previously in our laboratory [46]. Both solutions (Ch and FITC) were mixed, at room temperature (RT), under constant stirring, protected from light, for 3 h. Following this, FITC-labeled Ch was precipitated with 1.0 M NaOH and washed with Milli-Q water, until no fluorescence could be seen in the supernatant. FITC-labeled Ch was finally lyophilized, dried, weighed and stored in a dessicator, protected from light until use. FITC-labeled Ch was dissolved in 0.2 M AcOH to a 1% final concentration, and the solution was degassed and stored at 4 °C, protected from light until use. Fluorescently labeled Ch films were prepared by spincoating (SCS Cookson Electronics Spincoater model G3P-8) for 2 min at 9000 rpm to ensure a homogeneous distribution of the polymer. Briefly, 80 μl of the FITC-Ch solution was deposited onto round glass coverslips (13 mm diameter), which had been previously washed overnight with nitric acid 65%, washed several times with distilled water and finally calcinated overnight at 220 °C. The spinning procedure was repeated four times, in order to produce a four-layer film, so that FITC could be detected by fluorescence microscopy. Films were then neutralized with 0.1 M NaOH, washed twice with distilled water and dried overnight at 37 °C. Prior to cell culture, films were disinfected with filtered 70% ethanol, washed twice with PBS and incubated with complete culture medium for 1 h at 37 °C. Where appropriate, Ch films were further modified with Fg, by incubating them for 2 h with a Fg solution, as described above. Film thickness was determined using an imaging ellipsometer (EP3, Nanofilm), as previously described [47], and found to be 112.2 ± 4.8 nm ($n = 3$).

2.4. Monocyte isolation and osteoclast differentiation

Buffy coats (BCs) (kindly donated by Instituto Português do Sangue) from healthy blood donors, were used to isolate monocytes,

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