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In vitro evaluation of the risk of inflammatory response after chitosan/HA and chitosan/β-1,3-glucan/HA bone scaffold implantation

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ABSTRACT

The aim of the study was to evaluate *in vitro* the risk of inflammatory response induced by chitosan/hydroxyapatite (chit/HA) and novel chitosan/β-1,3-glucan/hydroxyapatite (chit/glu/HA) bone scaffolds. The inflammatory response was assessed *via* measurement of proinflammatory cytokine and ROI production by human monocytes, macrophages, and osteoblasts stimulated with investigated scaffolds. Moreover, adsorption of human serum/plasma proteins to the tested materials was determined.

Both biomaterials did not induce intracellular ROI generation by monocytes, macrophages, and osteoblasts and did not stimulate proinflammatory cytokine (IL-6 and TNF-α) production by inflammatory cells. Moreover, the chit/glu/HA material induced increased TNF-α production by osteoblasts that is believed to enhance osteogenic differentiation. Thus, it was demonstrated that chit/HA and chit/glu/HA scaffolds carry a low risk of biomaterial-induced inflammatory response and are promising materials as bone scaffolds for bone tissue engineering and regenerative medicine applications.

1. Introduction

Bone scaffolds for long-term implantation are usually selected by analysis of their mechanical properties, toxicity, and stimulation of the osteoblast adhesion, proliferation, and differentiation [1–3]. However biomaterials for bone tissue engineering and regenerative medicine applications are also expected to well integrate with the host tissue and not to induce foreign-body inflammatory response to the surface of implanted material [4].

Biomaterial-induced inflammation is the immune reaction of the body to implanted material at both cellular and molecular level [5]. In both cases, blood-contact and tissue-contact, shortly after implantation (seconds to minutes) the surface of the material is coated with plasma or tissue proteins that are recognized by the leukocytes [4,6–8]. 4 to 8 h later, host cells, mainly monocytes and some fibroblasts, migrate to the implant and adhere to the surface coated with plasma/tissue proteins [8]. Adhered monocytes differentiate to macrophages that may further form multinucleated foreign body giant cells [4,8]. Within few days, biomaterial surface is covered by a layer of fibrotic tissue (fibroblasts and collagen), macrophages and foreign body giant cells [8]. Extended inflammation (3–4 weeks) may lead to fibrous encapsulation and granuloma tissue formation resulting in failure of biomaterial integration [7].

The adherent phagocytes are involved in many implant-associated adverse reaction like inflammation surrounding biomaterial, oxidative degradation of the material, or fibrous thickening surrounding implants [8,9]. As protein adsorption to the biomaterial surface occurs meaningfully faster than the migration of leukocytes to the implantation area, the host inflammatory cells interact not with the material itself but with the adsorbed and partially denatured host proteins [6–9]. The predominant host proteins found on the biomaterial surface are albumin, fibrinogen, and immunoglobulin G [8]. Nevertheless, it is the spontaneous adsorption of fibrinogen that is critical to the induction of inflammation at the site of implantation [8–10].

The engagement of phagocyte Mac-1 integrin with P1 epitope of fibrinogen adsorbed to the material surface leads to the adhesion and activation of the host inflammatory cells [10]. Surface-activated phagocytes release many growth factors, proinflammatory and profibrotic cytokines like IL-1β, VEGF, TNF-α, MCP-1, MIP-1α, IL-2, IL-6, IL-4, and IL-10 [4,5]. Activated macrophages may also generate toxic reactive oxygen intermediates (ROIs) harming surrounding cells in the microenvironment and causing oxidative damage of the biomaterial [7]. While initial host inflammatory response is essential for normal wound healing process, the prolonged inflammation impairs regenerative capacity of the material and hinders new tissue formation [7,11].

Currently, an animal model is still the most commonly used method to study the host response to the implanted biomaterial [12]. However, recent progress in molecular biology techniques allows for *in vitro* biomaterial-induced inflammatory response evaluation using enzyme-linked immunosorbent assay (ELISA), RT- PCR, or multiplexed cytokine immunoassay [5]. Nevertheless there are still very few papers describing *in vitro* methods using cellular models to evaluate potential implant-induced inflammatory response [4,5].

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Our previous reports describe the fabrication and basic characterization of bi-component chitosan/hydroxyapatite (chit/HA) [2,13,14] and novel tri-component chitosan/linear bacterial β-1,3-glucan/hydroxyapatite (chit/glu/HA) scaffolds [1–3,14] for bone tissue engineering application. It was proved that fabricated biomaterials are non-toxic and favourable to osteoblast adhesion, growth, proliferation, and differentiation [1–3,13,14]. Although investigated materials were demonstrated to possess excellent biocompatibility (particularly chit/glu/HA scaffold) [1–3,13,14], their poor mechanical properties (relatively low Young's modulus values) [1,14] limit their applications to non-load bearing implantation area as cell scaffolds to fill small bone losses and to provide rapid osteoblast ingrowth and enhanced osteogenic differentiation thereby accelerating new bone formation at the site of implantation.

As inflammation is a significant problem for clinical application of biomaterials, presented work focused on *in vitro* evaluation of the risk of inflammatory response induced by chit/HA and novel chit/glu/HA scaffolds for bone tissue regeneration. The inflammatory response was assessed *via* measurement of proinflammatory cytokine and ROI production by human monocytes, macrophages, and osteoblasts stimulated with investigated scaffolds.

The linear bacterial β-1,3-glucan (curdlan) has a great potential in biomedical applications. So far, this polysaccharide was used as an antitumor and antibacterial agent [15] as well as effective drug carrier [15,16]. However, according to available literature apart from our previous articles there are no papers describing linear bacterial β-1,3-glucan (curdlan) as a constituent of cell scaffold for bone regeneration applications. As curdlan is known to be immune stimulating polymer capable of inducing inflammatory reactions by activation of macrophages [15–17], the aim of this study was also to prove that unlike curdlan solution, curdlan in its gelled form that is present in the chit/glu/HA scaffold does not stimulate inflammatory cells. Thus, human monocytes and macrophages were also treated with just curdlan component of the chit/glu/HA scaffold.

Presented studies also focused on the evaluation of adsorption of human serum and plasma proteins to the chit/HA and chit/glu/HA scaffolds. Moreover, relative fibrinogen adsorption was determined.

2. Materials and methods

2.1. Preparation of tested samples

The chitosan/hydroxyapatite (chit/HA) material was composed of 4.0 wt.% krill chitosan (Sea Fisheries Research Institute in Gdynia, Poland) and 80 wt.% hydroxyapatite (HA BIOCER bioceramics, Chema Elektromet, Rzeszow, Poland). The chitosan/bacterial β-1,3-glucan/hydroxyapatite scaffold (chit/glu/HA) was made of 2.0 wt.% krill chitosan, 8.0 wt.% linear bacterial β-1,3-glucan — curdlan (Wako Pure Chemicals Industries, Japan), and 80 wt.% hydroxyapatite. Scaffold fabrication was described previously [1,13]. Curdlan (β-1,3-glucan) sample was prepared similarly to the chit/glu/HA scaffold. Briefly, 8.0 wt.% curdlan solution prepared in distilled water was placed into cylinder-shaped mould and heated for 20 min at 90 °C in water bath to gel the sample.

Prior to all *in vitro* experiments, prepared cylinder-shaped materials were cut into discs approximately 1 mm thick and 5 mm in diameter and sterilized by ethylene oxide. Samples were then placed in 96-multiwell plates and preincubated overnight in a complete culture medium at 37 °C in the case of cell culture experiments or in phosphate buffered saline (PBS, Sigma-Aldrich Chemicals) in the case of protein adsorption test.

2.2. Cell culture experiments

The biomaterial-induced inflammatory response was assessed in *in vitro* conditions using human acute monocytic leukaemia cells (THP-1), human THP-1-derived macrophages and normal human foetal osteoblasts (hFOB 1.19) obtained from ATCC (American Type Culture Collection). THP-1 cell line is widely used as an *in vitro* model of inflammatory response as THP-1 monocytes and THP-1-derived macrophages were proved to closely resemble primary monocytes and primary monocyte-derived macrophages respectively, with regard to morphology, membrane receptor/antigen expression, and secretory products [18,19]. THP-1 cells were cultured in a RPMI-1640 medium (ATCC-LGC Standards) supplemented with 10% foetal bovine serum (FBS, PAA Laboratories), 0.05 mM mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich Chemicals) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. To induce differentiation of THP-1 monocytes into adherent macrophages, THP-1 cells were cultured for 3 days in the complete RPMI-1640 medium supplemented with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chemicals). Then, medium containing PMA was removed and PMA-treated cells were incubated in fresh complete RPMI-1640 medium for further 4 days to enhance monocytic differentiation [20].

HFOB 1.19 cells were cultured in a 1:1 mixture of DMEM/Ham F12 medium without phenol red (Sigma-Aldrich Chemicals) supplemented with 10% FBS, 300 μg/ml G418 (Sigma-Aldrich Chemicals), 100 U/ml penicillin, 100 μg/ml streptomycin and maintained at 34 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2.1. Measurement of reactive oxygen intermediates production

ROI generation by human monocytes (THP-1), THP-1-derived macrophages, and osteoblasts (hFOB 1.19) was measured by fluorometric method using 2′,7′-dichloroflurescin diacetate dye (DCF-DA, Sigma-Aldrich Chemicals). The cell membrane permeable DCF-DA is deacetylated by intracellular esterases to a non-fluorescent compound that is further oxidized by cellular ROIs into green fluorescent DCF that can be detected using fluorescence plate reader or microscopy.

ROI production was evaluated using fluid extracts of biomaterials prepared by immersing the test materials in Hanks` Balanced Salt Solution (HBSS, Sigma-Aldrich Chemicals) containing 10% FBS and incubating for 24 h at 37 °C. Extracts were prepared according to ISO 10993-5 [21] as it was described earlier [13].

Non-adherent THP-1 monocytes were centrifuged at 125 × *g* for 10 min to pellet the cells. Then, monocytes were resuspended in 10 ml of 25 μM DCF-DA solution prepared in complete RPMI-1640 medium and incubated for 1 h at 37 °C in the dark. Afterwards, cells were washed by centrifugation with PBS buffer and resuspended to 1×10^6/ml in HBSS containing 10% FBS — negative control, 100 μM H_2O_2 (Avantor Performance Materials Poland) prepared in HBSS with 10% FBS — positive control, and in extracts of tested samples: curdlan gel, chit/HA, and chit/glu/HA scaffolds. THP-1 monocytes were seeded in black, clear bottom 96-multiwell plates in 100 μl at a concentration of 1×10^5 cells/well and incubated for 3 h at 37 °C. Plates were read using Bio Tek Synergy H4 Hybrid Microplate Reader with the excitation wavelength at 485 nm and emission wavelength at 528 nm (area-scan readings were recorded).

For monocytic differentiation, THP-1 cells were seeded in black, clear bottom 96-multiwell plates at a concentration of 1×10^5 cells/well and stimulated with PMA as described in section 2.2. Then, the culture medium was removed and THP-1-derived adherent macrophages were loaded with 25 μM DCF-DA solution prepared in complete RPMI-1640 medium (100 μl/well) for 1 h at 37 °C in the dark. Afterwards, DCF-DA solution was removed, cells were washed with PBS buffer and 100 μl of the appropriate extracts were added. HBSS containing 10% FBS served as negative control of ROI production and 100 μM H_2O_2 solution was used as ROI inducing agent. Plates were incubated for 3 h at 37 °C and fluorescence intensity was measured using microplate reader.

Human osteoblasts (hFOB 1.19) were seeded in black, clear bottom 96-multiwell plates in 100 μl complete culture medium at a concentration of 1.5×10^4 cells/well. After 24-hour incubation at 34 °C the growth medium was gently discarded and cells were loaded with 25 μM DCF-DA solution prepared in complete DMEM/Ham F12 medium (100 μl/well) for