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The response of macrophages to titanium particles is determined by macrophage polarization

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ABSTRACT

Aseptic loosening of total joint replacements is driven by the reaction of macrophages to foreign body particles released from the implant. It was hypothesized that the macrophages' response to these particles is dependent, in addition to particle characteristics and contaminating biomolecules, on the state of macrophage polarization as determined by the local cytokine microenvironment. To test this hypothesis we differentiated M1 and M2 macrophages from human peripheral blood monocytes and compared their responses to titanium particles using genome-wide microarray analysis and a multiplex cytokine assay. In comparison to non-activated M0 macrophages, the overall chemotactic and inflammatory responses to titanium particles were greatly enhanced in M1 macrophages and effectively suppressed in M2 macrophages. In addition, the genome-wide approach revealed several novel, potentially osteolytic, particle-induced mediators, and signaling pathway analysis suggested the involvement of toll-like and nod-like receptor signaling in particle recognition. It is concluded that the magnitude of foreign body reaction caused by titanium particles is dependent on the state of macrophage polarization. Thus, by limiting the action of M1 polarizing factors, e.g. bacterial biofilm formation, in peri-implant tissues and promoting M2 macrophage polarization by biomaterial solutions or pharmacologically, it might be possible to restrict wear-particle-induced inflammation and osteolysis.

1. Introduction

Total hip replacement is an effective method for treating end-stage degenerative and inflammatory joint diseases [1]. Short- and mid-term outcomes of total joint replacement operation are generally excellent and consequently demand for total hip replacement surgery has been increasing steadily. Long-term survival of the prosthesis is, however, limited, and the number of revision total hip replacement operations is increasing [2]. The main reason for these long-term prostheses failures and revision operations is aseptic loosening. The osteolysis and implant loosening are driven by implant micromotion, pressure waves of the pseudosynovial fluid, stress shielding, and by chronic inflammatory reaction caused by implant-derived biomaterial particles [3]. These particles are generated due to abrasion between prosthesis-bearing surfaces or, as in the case of titanium particles, are released from the osteoconductive porous bone–prosthesis interface or due to wear between the various components of modular implants [4].

According to the established "particle disease" model of aseptic osteolysis, wear particles in the interface tissue, developing around loosening prostheses, are phagocytosed by macrophages [5]. In response to foreign body particulate material, macrophages produce inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, PGE2), chemokines (e.g. CCL2, CCL3, IL-8) and growth factors (M-CSF, GM-CSF, VEGF) [6,7]. These factors lead to further recruitment of monocyte macrophages into the interface tissue and directly support osteoclastogenesis and osteoclast function. Furthermore, macrophage-derived inflammatory mediators also increase the production of receptor activator of nuclear factor-kB ligand (RANKL) and decrease the production of osteoprotegering (OPG) from local stromal cells [8]. Increased RANKL production along with the inflammatory cytokines and decreased osteoprotegerin (OPG) production creates a microenvironment which favors osteoclastogenesis and osteolysis, finally leading to prosthesis loosening [9].

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Wear particle recognition and subsequent macrophage activation to inflammatory phenotype are thus considered as fundamental events in the pathogenesis of aseptic osteolysis. Numerous early studies have characterized the effects that the physical and chemical properties of wear particles have on macrophage activation [3–6]. Since then, several in vitro and in vivo studies have shown that wear particles of various natures are in fact relatively inert, and that several of their previously observed inflammatory and osteolytic characteristics are better explained by the various biomolecules adhering to their surfaces [10,11]. In particular, endo- or exogenous danger signal molecules (DAMPs), e.g. bacterial lipopolysaccharide (LPS), have been shown to mediate wear-particle-induced macrophage activation via recognition by macrophages germ line encoded pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) [11,12].

In the field of immunology it has become increasingly clear that macrophages represent heterogeneous and adaptive cell populations, readily capable of adopting different phenotypes and activation states as a response to multitude of signals derived from the local microenvironment [13–15]. Reflecting the Th1/Th2 polarization paradigm of T lymphocytes, a concept of M1 and M2 macrophages or "classically" and "alternatively" activated macrophages, respectively, has been established [16]. M1 polarization is induced by Th1 signature cytokine IFN-γ and/or TLR signaling, which up-regulate genes involved in cell mediated immunity, killing of phagocytosed pathogens, effective antigen presentation and production of a distinct array of pro-inflammatory cytokines and chemokines [13,16]. Alternative macrophage activation referred originally to a macrophage phenotype induced by exposure to Th2 signature cytokines IL-4 or IL-13 [17]. This M2a macrophage phenotype is characterized by suppression of pro-inflammatory cytokine production, a suppressed ability to kill intracellular pathogens and suppressed antigen presentation ability, and further by production of anti-inflammatory mediators and distinct set of chemokines [18–21]. In M2a macrophages membrane receptors with a scavenger function are up-regulated, as are a variety of molecules involved in tissue regeneration, wound healing, granuloma formation and immunity against larger parasites. Since the original description of alternative macrophage activation, several distinct macrophage phenotypes, induced by recognition of immune complexes in combination with TLR stimulus (M2b) or by exposure to IL-10, TGF-β or glucocorticoids (M2c), have been characterized [20]. Collectively these macrophages are characterized by high levels of IL-10 production and are primary considered to perform immunosuppressive or modulatory functions [20].

The concept of macrophage polarization and plasticity has received little attention in the context of wear particle recognition and aseptic osteolysis. This is somewhat surprising, considering, for instance, that M1 and M2 macrophages express different levels of PRRs and have fundamentally different abilities to produce pro-inflammatory and chemotactic mediators [21]. It was thus hypothesized that the macrophage response to wear particles is dependent not only on the particles' physical and chemical characteristics or opsonization by DAMPs, but also on the state of macrophage polarization. To test this hypothesis, we differentiated and polarized M1 and M2 macrophages in vitro from human peripheral blood monocytes and compared their responses to endotoxin-free titanium particles using genome-wide microarray analysis and multiplex cytokine assay.

2. Materials and methods

2.1. Particles and LPS

Titanium particles, with a mean diameter of 3.7 ± 1.8 μm as determined by scanning electron microscopy, were bought from Alfa Aesar (Ward Hill, MA, USA). To remove any LPS contamination the particles were washed following protocol by Ragab et al. [22]. Briefly, the particles were incubated in 25% nitric acid for 20 h at room temperature followed by 20 h incubation in 0.1 N NaOH in 95% ethanol. Between the treatments, particles were washed twice with phosphate-buffered saline (PBS, pH 7.4). After five cycles of washes, particles were resuspended in 720 mg ml^{-1} PBS and stored at +4 °C until use (particle stock). LPS decontamination was confirmed using Limulus amebocyte lysate chromogenic endpoint assay kit (Hycult Biotech, Uden, the Netherlands) following the manufacturer's instructions. Particle LPS levels were below the assay's 0.01 EU ml^{-1} detection limit. Immediately before being applied to the macrophage cultures, particle stock was vigorously vortexed for 2 to 3 min, after which appropriate volume of particle stock was diluted and mixed with complete cell culture medium so that final particle concentration of the medium was 6×10^6 particles ml^{-1}.

2.2. Monocyte isolation and macrophage differentiation

The human macrophages used in the study were initially isolated from buffy coats (provided by Finnish Red Cross Blood Service) of four blood donors. Three of the donors were women (ages 21, 49 and 58 years) and one was a man (aged 58). Donors had no major illnesses or medications. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using the Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) density gradient technique. $CD14^+$ monocytes were purified from PBMC by positive selection using the MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany). $CD14^+$ cells were counted using a Z1 Particle Counter (Beckman Coulter, Indianapolis, IN, USA) and pipetted into tissue-culture-treated 24-well plates (BD, Franklin Lakes, NJ, USA), 3×10^5 cells per well, in 0.5 ml of Gibco RPMI-1640 GlutaMAX-1 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (complete medium). Cells were allowed to adhere for 1 h in a 5% CO_2 incubator at +37 °C, after which non-adherent cells were removed by rinsing with PBS. Adherent monocytes were differentiated to M0 macrophages by culturing them for 7 days in humidified 5% CO_2-in-air at +37 °C in the complete medium supplemented with 100 ng ml^{-1} of macrophage colony stimulating factor (M-CSF, R&D systems, Minneapolis, MN, USA).

2.3. Macrophage polarization and particle stimulation

Following macrophage differentiation, macrophages were polarized by one day's culture in complete medium supplemented with 100 ng ml^{-1} of M-CSF (M0 polarization), 20 ng ml^{-1} IFN-γ (M1 polarization), or 20 ng ml^{-1} IL-4 (M2 polarization) (all from R&D systems) [23]. The polarizing medium was then removed and complete medium with titanium particles was added to the M0, M1 and M2 macrophages, approximately 10 particles per macrophage, while corresponding control cells received particle-free complete medium. After 4 or 24 h of particle stimulation, cell culture supernatants were collected, kept on ice, centrifuged and stored at −75 °C until used. Immediately after supernatant collection, cells were rinsed with PBS and total RNA was extracted using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). Total RNA was stored at −75 °C until used. 4 h RNA samples were used for microarray and quantitative RT-PCR analyses and 24 h supernatant samples for cytotoxicity assay and protein suspension arrays. Additional RNA samples were collected after one day of macrophage polarization, prior to particle stimulation, and these samples were used to confirm macrophage phenotype by qRT PCR.