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Different activation of monocyte-derived macrophages by antimicrobial peptides at a titanium tibial implantation in rabbits

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ABSTRACT

The aim of our study was to assess the functional and morphological features of monocyte derived Mfs from rabbits with titanium (Ti) tibial implants, their ability to polarize towards M1 or M2 and their reactivity after stimulation with neutrophil antimicrobial peptides extract (AMP). The study was conducted on six White New Zealand rabbits with tibial implants of pure titanium Grade 2. Blood was taken before implantation and 14 days after biomaterial implantation. Then, blood-derived Mfs were cultured and their function was assessed on the basis of morphological changes, generation of nitric oxide (NO), superoxide, and proteases release after treatment with LPS or dexamethasone (Dex). As a result of polarization we specified two subpopulations of Mfs with features characteristic of each subtype. M1 stimulated with LPS exhibited an increased NO and superoxide level, and M2 stimulated with Dex which showed higher arginase and lower free radical generation. Addition of AMP evoked further changes in Mfs morphology and function. After 24 h stimulation with AMP an increase of NO was observed in all cultures, whereas after 48 h it decreased. Production of superoxide lowered, especially after 48 h, when M1 generated 6.00 ± 0.2 nM and M1/AMP culture generated 5.2 ± 0.1 nM of superoxide. Our study revealed that activated Mfs stimulated with AMP demonstrated both pro- and anti-inflammatory features. Moreover, we did not detect significant differences between the response of Mfs cultured from blood derived monocytes before and after implantation of Ti implants.

1. Introduction

Macrophages (Mfs) are the subpopulation of leucocytes essential for regeneration, repair and remodeling in numerous tissues (Novak and Koh, 2013a). Differentiated tissue Mfs are derived both from circulating monocytes and tissue resident cells (Eligni et al., 2013). Blood-derived Mfs and their precursor monocytes as key members of innate immunity first recognize and attack different pathogens, then this activity is combined with secretion of inflammatory mediators to initiate inflammation (Ma et al., 2014). However, chronic inflammation and persistent Mfs accumulation are often associated with tissue destruction. These contradictory roles of Mfs in tissue injury and repair are related to the ability of Mfs to form markedly different phenotypes in response to specific environmental cues. Mfs have long been known to assume a proinflammatory phenotype termed “classically activated” or M1, in response to, among others, lipopolysaccharide (LPS) and “alternatively activated” or M2 phenotype. On the basis of the stimulus from the microenvironment, M2 Mfs were divided into subtypes, namely M2a/b/c. *In vitro* M2a phenotype is produced by exposure to IL-4 or Il-13, M2b phenotype is obtained by treatment with a combination of IgG-immune complexes with LPS, and M2c are generated under the influence of glucocorticoids. Both M2a and M2c subpopulations not only cause suppression of inflammation but also promote tissue repair (Das et al., 2015; Novak and Koh, 2013a, 2013b). However, a damaged tissue contains a myriad of factors that could influence Mfs behavior in unexpected ways, and the actual factors influencing Mf phenotype during *in vivo* tissue injury and repair remain largely undetermined (Novak and Koh, 2013a).

In the light of the fact that pathogenic colonization and post-operative infection is a serious risk associated with the implantation of orthopedic implants we decided to apply titanium (Ti) implants coated with silver. This addition provides better resistance to pathogenic colonization (Cavanaugh et al., 2016). To enhance antimicrobial effect some authors applied antimicrobial peptides, *i.e.* cathelicidins.

Cathelicidins are host defense peptides which besides their direct antimicrobial action display multiple functions relating to tissue repair and innate immunity. For example, human cathelicidin LL-37 is involved in healing of human wounds, wound closure and rebuilding

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of vascular structures together with re-epithelialization. The pro-healing effects of cathelicidins seem to be mediated through modification of growth-factor/receptor interactions and angiogenesis (Wu et al., 2010). The role of cathelicidin in the enhancement of bone healing has not been thoroughly evaluated to date. Previously it has been revealed that synthetic variant of murine cathelicidin CRAMP-derived peptide (termed AS 10) has a broad spectrum of biofilm-inhibitory activity against *C. albicans* and various gram positive and gram negative bacteria. Moreover, it does not have adverse effects on viability or functionality of diverse human cell types involved in bone formation. Thus, AS10 is an interesting product for the development of a biofilm-preventive coating for Ti implants (De Brucker et al., 2014).

An earlier report indicated that the effects of cathelicidin on Mfs response may vary depending on the exogenous and endogenous origin of peptide and the prior activation state of the cell (Pinheiro da Silva et al., 2009). Previously we prepared the neutrophil antibacterial peptides extract (AMP extract) from rabbit blood containing cathelicidins and defensins, and we examined *in vitro* the influence of this extract on some inflammatory cells, namely neutrophils and Mfs, as a preliminary study (Szponder et al., 2017).

Recent data raise the possibility of manipulating Mfs function by various pharmacological means with the goal of enhancing tissue healing in many pathological conditions (Mantovani et al., 2013). Tissue Mfs, however, are not easily obtained and handled without affecting viability. Thus, monocyte derived macrophages (MDM) are commonly accepted as a good surrogate of Mfs infiltrating tissues (Eligni et al., 2013). Apart from the phenotypic manipulations of endogenous Mfs to enhance tissue healing conducted on animal models, several studies have indicated that cell therapy with exogenous Mfs can promote healing in a variety of tissues, and prior *ex vivo* activation of these Mfs to M1, M2a or other phenotype may alter their ability to promote repair. Treatment with exogenous M1 bone-marrow derived Mfs enhances muscle regeneration and reduces fibrosis after injury (Novak and Koh, 2013b). Moreover, activated Mfs suspension (containing M1 polarized Mfs) was previously prepared from human MDM and stimulated *ex vivo* for treatment of infarcted heart on the rat model (Leor et al., 2006). These authors assumed that local delivery of prepared Mfs might modulate local inflammatory response, suppress local injury, and promote tissue vascularization, healing and repair. Better understanding of Mfs activation and its regulation during tissue repair may help to develop new therapies in which manipulation of Mfs function can be used to boost healing (Novak and Koh, 2013b).

There are some reports concerning the *in vitro* activity of Mfs in contact with Ti implant (Refai et al., 2004; Tan et al., 2006; Lee et al., 2011; Ma et al., 2014; Moura et al., 2014), but there is a lack of publications concerning the influence of biomaterial implantation on the activity of MDM from implanted subjects. Thus, our first aim was to assess the functional and morphological features of MDM from rabbits with Ti implants with silver surface in tibia and their ability to polarize towards M1 or M2. Our second aim was to evaluate the influence of the autologous AMP extract on MDM for the possible clinical use in the enhancement of healing.

2. Materials and methods

2.1. Animals

The study was carried out on 6 healthy New Zealand White (NZW) rabbits, males, 7–9 months old, with the body weight between 3500 and 4000 g. The rabbits were individually caged, fed with a standard balanced rabbit chow and provided with water *ad libitum*. All animals were treated according to the guidelines for laboratory animal treatment and care. The study protocol was approved by the Local Ethics Committee of the University of Life Sciences in Lublin and the experiment was performed in compliance with animal protection regulations.

Complete blood cells count analysis was performed on whole blood using the Vet EXIGO analyser (Boule Medical AB) before the start of the experiment. In all the rabbits involved in the study haematological values were within the normal range.

2.2. Preparation of neutrophil crude extract of AMP

The procedure of AMP extract preparation was conducted two weeks before the start of the experiment with implantation of Ti implants. For isolation of neutrophils 2–3 ml of blood were collected from the ear vein of each experimental rabbit into the syringe with an anticoagulant 3.8% citrate. After the red blood cells were lysed by addition of 0.83% ammonium chloride at the ratio of 3:1 to the obtained blood sample, the remaining pellet was washed twice with phosphate-buffered saline (PBS-Biomed, Lublin, Poland). The final cells (> 85% of PMN on the May-Grunwald-Giemsa-stained preparations) were then homogenized to release the neutrophil granules. These granules were collected (25,000 × g, 40 min, 4 °C), suspended in 10% acetic acid and stirred overnight at 4 °C to extract the antimicrobial peptides. The solution containing the peptides was separated from the granules (25,000 × g, 20 min, 4 °C) and the obtained extract was considered as AMP neutrophil extract. The portions of 40 μg/ml of this extract were lyophilized and stored at − 20 °C for further experiment. These lyophilizates were dissolved in PBS and used for stimulation of Mfs cultures. As it has been estimated in our previous study this extract cathelicidins, 15,000 Da antimicrobial peptide, cathelin-like fragments, CAP 18, and rabbit defensins, namely NP-1, NP-2, NP-3a, NP-3b, and NP-5 (Szponder et al., 2017).

2.3. Description of titanium implants

Commercially pure titanium Grade 2 discs were prepared and suited for our experiment. The surface of the implants was pre-treated with a triple surface etching formula (TSE). To obtain titanium implants modified with silver nanoparticles silver incorporation on the titanium surface was done using Tollens method. A detailed description of the implants preparation method and characterization of their properties have been described previously (Pokrowiecki et al., 2015).

2.4. Surgical procedure

A physical examination of each rabbit was performed before the surgical procedure. After premedication with xylazine (Sedazin; Biowet, Pulawy, Poland) 5 mg/kg and ketamine (VetaKetam; Vetagro, Lublin, Poland) 30 mg/kg intramuscularly, the marginal ear vein was catheterized with a 22-gauge sterile catheter for the administration of anesthesia. The animals received ketamine (0.35 mg/kg/min) intravenously at a continuous rate infusion diluted with 5% of glucose. The procedures were performed under standard sterile conditions. After hair removal, shaving, disinfection and draping, a straight 3-cm skin incision was made over the medial proximal tibia. Then, after surgical exposure, the 4 mm defect was made using an electric surgical drill. During drilling, the drill was continuously cooled with saline. Just before insertion of the implants, the hole was irrigated with saline to remove any bone shards. Titanium implant was placed into the perforation and pressed into surgical cavity until it was fixed to the cortical bone (Fig. 1). Then, the muscle tissue and skin were sutured. After the surgery, the rabbits were inspected daily for clinical signs of complications or adverse reactions.

2.5. Monocyte isolation and generation of monocyte-derived macrophages (MDM)

Blood was drawn before the surgical procedure (1st time point) and then two weeks after biomaterial implantation (2nd time point). Peripheral blood mononuclear cells (PBMC) were separated by gradient