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Biodegradation of metallic magnesium elicits an inflammatory response in primary nasal epithelial cells ☆

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ABSTRACT

Resorbable magnesium-based implants hold great promise for various biomedical applications, such as osteosynthesis and coronary stenting. They also offer a new therapeutic option for the treatment of chronic rhinosinusitis, but little data is yet available regarding the use of magnesium in the nasal cavity. To model this field of application, primary porcine nasal epithelial cells were used to test the biocompatibility of degrading pure magnesium and investigate whether the degradation products may also affect cellular metabolism. Magnesium specimens did not induce apoptosis and we found no major influence on enzyme activities or protein synthesis, but cell viability was reduced and elevated interleukin 8 secretion indicated proinflammatory reactions. Necrotic damage was most likely due to osmotic stress, and our results suggest that magnesium ion build-up is also involved in the interleukin 8 release. Furthermore, the latter seems to be mediated, at least in part, by the p38 signaling pathway. These effects probably depended on the accumulation of very high concentrations of magnesium ions in the in vitro set-up, which might not be achieved in vivo, although we cannot exclude that further, as yet unknown, factors played a role in the inflammatory response during the degradation process. In conclusion, the biocompatibility of pure magnesium with cells in the immediate vicinity appears less ideal than is often supposed, and this needs to be considered in the evaluation of magnesium materials containing additional alloying elements.

1. Introduction

Magnesium-based resorbable implants have emerged as one of the most intriguing fields of biomedical research [1–4]. These implants are intended to slowly degrade after having fulfilled their temporary function of supporting tissue during the healing and remodelling process, thus obviating the need for implant removal surgery [5]. In addition, it has been reported that magnesium has some unique advantages over conventional implant materials, e.g. favorable mechanical properties and potential osteoconductive effects [2,3].

Because of the biocompatibility demands of biomaterials, the degradation needs to occur without detrimental effects on any part of the organism. Magnesium itself, representing the main constituent of the alloys, is considered to be safe because it is abundantly present in the body and it is suggested that any surplus of magnesium released during implant corrosion can be easily excreted via the kidneys [2]. Plasma magnesium levels and organ functions hence remain unchanged [6]. However, a significant increase in extracellular magnesium concentration is anticipated in the immediate vicinity of an implant, which is likely to be accompanied by alkalinization and hydrogen evolution on the biomaterial's surface [7]. These milieu changes may affect the cells at the tissue–implant interface, reducing their viability and/or triggering an inflammatory reaction. Therefore, the local tissue tolerance of corroding magnesium is not as self-evident as it may seem, and a very recent in vivo study reported various local pathological effects with severe bone affections after long-term implantation of the magnesium alloy ZEK100 [8].

Biomedical magnesium alloys are predominantly designed for orthopaedic and cardiovascular applications, but the nasal cavity has recently been proposed as another potential target [9,10]. Chronic rhinosinusitis (CRS) patients often suffer from stenosis of the paranasal sinus apertures, and the formation of scar tissue is a common complication after surgical intervention. Silicon stents are frequently used to maintain the ventilation of the sinuses during the wound healing period, but the outcome may be unsatisfactory. Depending on the duration of stenting, typical problems include restenosis, stent dislocation or infection, nasal congestion, unpleasant odour and renewed tissue trauma when the silicon

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material is finally removed [11–13]. A resorbable magnesium-based stent may overcome some of these limitations, thus presenting a therapeutic option in the treatment of CRS [10].

The airway epithelial cells represent the cell type in direct contact with an implant in the nasal cavity. In order to simulate this situation we decided to assess the biocompatibility of magnesium by utilizing a model of primary porcine nasal epithelial cells (PNEC), because the pig is considered a suitable animal model for future in vivo studies of degradable paranasal sinus stents.

Apart from biocompatibility aspects, the local surplus of magnesium might also exert some effects on metabolic processes. Magnesium is an essential element in the body with diverse cellular functions as a regulator of ion channels, a stabilizer of membranes and nucleic acids, a co-factor of hundreds of enzymes and, in the form of $MgATP^{2-}$, the substrate for energy-consuming reactions [14]. Moreover, magnesium ions have been described as a regulator of protein synthesis and proliferation [15]. But whether these parameters experience any changes due to the presence of degrading magnesium has not been studied in any detail. Therefore, the aim of this study was to elucidate the more basic mechanisms and cellular reactions to the biodegradation of metallic magnesium. We did not find any remarkable metabolic effects, but we did observe an as-yet-unreported induction of the proinflammatory cytokine interleukin 8.

2. Material and methods

2.1. Material preparation

Magnesium (99.92% pure; Magnesium Elektron UK, Manchester) was processed by gravity die casting. The material was melted at a temperature of 750 °C in a steel crucible and subsequently stirred for 30 min in a protective argon atmosphere (gas supplied at 2 l min^{-1}; Air Liquide, Duesseldorf). The die-sets had a diameter of 130 mm and were preheated to 450 °C. After casting and before further processing, the pores and impurities that had resulted from the casting process, were abscised from the alloy billets. In order to obtain fine-grained alloys with advanced corrosive and mechanical properties, hot extrusion was carried out after casting using a 10MN extruder (SMS Meer, Moenchengladbach). The extrusion die, which had an orifice diameter of 10 mm, and the recipient were heated to 380 °C. Afterwards the respective cast billets were inserted into the extruder and pressed through the extrusion die with a profile velocity of 1.9 m min^{-1}. The deformation was realized by an extrusion ratio of 144. The magnesium material resulting from this process degrades relatively quickly during the first 48 h of immersion in modified simulated body fluid, exhibiting a mass loss of about 6.7%, as reported previously [16].

For this study, test specimens with a height of 2 mm and a diameter of 5 or 8 mm were manufactured by machining. These specimens were degreased with ethanol and heat-sterilized at 180 °C for 2 h before use in the experiments.

Degradation media were prepared by incubating a magnesium specimen of 8 mm in diameter in 1, 3, 10, 20 or 30 ml of serum-free airway epithelial cell growth medium (AECGM, magnesium content 0.6 mM; Provitro, Berlin or PromoCell, Heidelberg) for 5 days at 37 °C in either a waterbath or an incubator in a 5% CO_2 atmosphere. Afterwards, the degradation media were filtered using a syringe filter (Minisart; Sartorius Stedim, Göttingen).

2.2. Cell culture

PNEC were isolated using a modified protocol according to Mao et al. [17]. In short, nasal septum and ventral turbinates were taken from pigs (euthanized for reasons not related to this study) and transported in Hanks' balanced salt solution (PAA, Pasching) on ice. Nasal mucosa was then removed from underlying tissue, washed five times in M199 medium supplemented with 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 250 ng ml^{-1} amphotericin B (all from PAA), hereafter referred to as M199+, cut into small pieces and incubated in M199+ containing 0.6 mg ml^{-1} protease type XIV (Sigma-Aldrich, Steinheim) at 4 °C overnight. Cells were collected by gentle agitation in M199+ containing 10% fetal calf serum (FCS; PAA) and preincubated in a Petri dish for 1 h to reduce fibroblast contamination. Afterwards, non-adherent cells were collected with the supernatant, washed three times in M199 + and resuspended in AECGM supplemented with antibiotics/antimycotics as above, 5% FCS and 10^{-7} M retinoic acid (AppliChem, Darmstadt) and either seeded immediately or cryopreserved in liquid nitrogen for later use. The epithelial origin of the cells was verified by immunocytochemical staining using antibodies against cytokeratin (clone C-11) or vimentin (clone V9; both Sigma-Aldrich), respectively, and a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (AbD Serotec, Düsseldorf).

Cells were seeded in 6-, 12- or 96-well plates (Greiner Bio-One, Frickenhausen) coated with rat-tail collagen (Roche, Mannheim) at a density of ~75,000 cells cm^{-2}. The medium was changed after 48 h, then every 2-3 days until confluency. The medium was then changed to serum-free conditions and culture was continued for 24 h before experiments were started.

2.3. Air–liquid interface (ALI) culture

In order to mimic the in vivo situation, PNEC were differentiated in vitro using an ALI. Cells were seeded on 6-well polycarbonate membranes (Nunc, Langenselbold) coated with rat-tail collagen at a density of ~150,000 cm^{-2} and cultured for 6 days with a medium change every other day before the medium was removed from the upper compartment to create the ALI. The medium was changed to serum-free conditions and cultivation was continued for 14 days to enable a differentiation of the cells before experiments were started. Differentiation was verified by immunocytochemical staining of β-tubulin (clone TUB 2.1; Sigma-Aldrich).

2.4. Treatment

For the experiments, the holding medium was removed and fresh medium (1 ml for the 12-well plates, 2 ml for the 6-well plates), fresh medium with additional magnesium chloride ($MgCl_2 \cdot 7H_2O$; Merck, Darmstadt) or degradation medium was added to the cells. Magnesium specimens (5 mm for the 12-well plates, 8 mm for the 6-well plates) were placed in inserts (Greiner Bio-One, Frickenhausen or SPL Life Sciences, Pocheon, respectively) before addition to the cells to avoid mechanical damage. In the ALI experiments where specimens were placed directly on the insert membrane with the cells, a pure titanium specimen of the same diameter and weight served as the vehicle treatment control, since titanium is supposed to be an inert biocompatible material. Further treatments in certain subsets of experiments included the addition of lipopolysaccharide (LPS 0111:B4; Sigma-Aldrich) as a positive control for cytokine release, medium containing magnesium oxide particles (MgO, 325 mesh; Sigma-Aldrich), medium with elevated pH or the regular supplementation of NaOH, respectively. In order to simulate the release of hydrogen from the degrading implants, an 8 mm specimen was placed in a sterile syringe filled with 2 ml of AECGM and the evolving hydrogen gas was passed into the culture medium via a FEP tubing (CMA Microdialysis AB, Stockholm).

Incubation was then continued for 24 or 48 h. After this period, the specimens were removed and the supernatants were collected