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Cytotoxic effect of galvanically coupled magnesium–titanium particles

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ABSTRACT

Recent work has shown that reduction reactions at metallic biomaterial surfaces can induce significant killing of cells in proximity to the surface. To exploit this phenomenon for therapeutic purposes, for example, for cancer tumor killing or antibacterial effects (amongst other applications), magnesium metal particles, galvanically coupled to titanium by sputtering, have been evaluated for their cell-killing capability (i.e. cytotoxicity). Magnesium (Mg) particles large enough to prevent particle phagocytosis were investigated, so that only electrochemical reactions, and not particle toxicity per se, caused cytotoxic effects. Titanium (Ti) coated magnesium particles, as well as magnesium-only particles were introduced into MC3T3-E1 mouse pre-osteoblast cell cultures over a range of particle concentrations, and cells were observed to die in a dosage-dependent manner. Ti-coated magnesium particles killed more cells at lower particle concentration than magnesium alone ($P < 0.05$), although the pH measured for magnesium and magnesium–titanium had no significant difference at similar particle concentrations. Complete cell killing occurred at 750 μg/ml and 1500 μg/ml for Mg–Ti and Mg, respectively. Thus, this work demonstrates that galvanically coupled Mg–Ti particles have a significant cell killing capability greater than Mg alone. In addition, when the pH associated with complete killing with particles was created using NaOH only (no particles), then the percentage of cells killed was significantly less ($P < 0.05$). Together, these findings show that pH is not the sole factor associated with cell killing and that the electrochemical reactions, including the reduction reactions, play an important role. Reduction reactions on galvanically coupled Mg–Ti and Mg particles may generate reactive oxygen intermediates that are able to kill cells in close proximity to the particles and this approach may lead to potential therapies for infection and cancer.

Statement of Significance

This paper demonstrates that during active corrosion of both Mg and Mg–Ti particles cells cultured with the particles are killed in a dose-dependent particle concentration fashion. Additionally, galvanically-coupled magnesium–titanium microparticles kill cells more effectively than magnesium particles alone. The killing effect was shown to not be due to pH shifts since no differences were seen for different particle types and pH adjusted medium without particles did not exhibit the same level of killing. The significance of this work is the recognition of this killing effect with Mg particles and the potential therapeutic applications in infection control and cancer treatment that this process may provide.

1. Introduction

Magnesium and its alloys are currently being widely investigated as potential degradable biomaterials, especially in orthopedic and cardiovascular applications [1–4]. Pure magnesium metal was used clinically as early as in the late 1800s, when magnesium was used as ligatures to stop bleeding, connectors to treat vessel anastomosis, and fixtures to treat bone fractures [5]. Many physicians noticed that magnesium was very biocompatible, allowing the bones and soft tissues to heal without any major adverse effects [5]. In fact, many investigators noticed that insertion of magnesium in surgical sites prevented infections, even when the operation was performed under non-sterile conditions [5]. Magnesium even

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showed potential in treating tumors, mostly subcutaneous hemangioma, in children [5–7]. Further investigations of magnesium recently showed that insertion of magnesium rods at bone fracture sites increases the rate of bone mineralization during magnesium corrosion [8–10]. This has been speculated to be due to the magnesium ions playing an important role in increasing osteoblast activity, since magnesium ion is an essential active-site component of many enzymes [8,11–15]. However, despite its desirable qualities discovered early on, magnesium as a biomaterial was discarded until recently because of its fast and unpredictable corrosion rate in vivo mainly depending on the size of the implant, chloride-containing environment, purity of the material (alloying elements), and the implant site (tissue type and degree of vascularization) [16–17]. Therefore, many studies began to focus on the alloying of magnesium, coating the metal surface, or treating the metal to increase the ductility and slow the corrosion rate [18–20].

Toxicity of magnesium ions has been studied and is considered very biocompatible with little adverse effects. Magnesium corrodes and releases magnesium ions, which are already present in the body in large amount (fourth most abundant ion in the body), with approximately 21–28 g of magnesium ions stored in an average body, more than half is stored in bone and the rest in muscle and soft tissue, and so the release of magnesium ions rarely causes any toxicity [21–22]. The corrosion of magnesium may rapidly release unwanted by-products in high concentrations, such as hydrogen gas, which may lead to dissociation and even necrosis of the tissues, and also hydroxide ions, which leads to the increase of local pH [23]. However, hydrogen gas usually disappears over time or can be quickly removed with syringe or incision [23–24]. The initial increase of pH can also have an adverse effect, but pH usually recovers rapidly, due to the body's natural buffer system [23,25]. The in vitro cytotoxicity of magnesium is mainly attributed to the increase of pH or very high concentrations of magnesium ions, which will have more significant adverse effects in vitro since the closed system does not offer any means to buffer the pH or eliminate excess magnesium ions or other metal elements alloyed to magnesium [26–27].

In addition to the oxidation products (ions and hydroxide), there are reduction reactions that must be present to balance all of the oxidation taking place. Reduction reactions in vivo are thought to be dominated by reduction of oxygen and reduction of water, both of which ultimately generate hydroxide, and potentially hydrogen gas. However, these reduction reactions also have intermediate products that include hydrogen peroxide, hydroxyl radical, and other chemical species that may have significant biological effects [28]. Thus, high corrosion rates result in high rates of generation of these reduction intermediates and it may be these species that are affecting cell viability during corrosion [29–31].

When highly corrodible magnesium alloys are galvanically coupled to a metal like titanium whose open circuit potential is a volt more positive than magnesium, two effects result. First, the rate of corrosion of the magnesium is galvanically increased, and second, the reduction reactions are focused onto the titanium surface (i.e. spatially separated from the oxidation). It is hypothesized that galvanic couples of magnesium and titanium will result in a significant enhancement in cell-killing ability.

This study proposes that galvanically coupled particles consisting of magnesium and titanium in direct contact with one another could be used therapeutically to deliver a killing effect in proximity to the particles. This could be used, for example, to treat tumors or local infections. Thus, the goal of this study is to show that magnesium galvanically coupled to titanium will be more effective in killing cells than magnesium alone, by giving magnesium or magnesium–titanium microparticles to cells in dose-dependent manner. This study will also show that pH is unlikely to be the sole factor in killing cells in vitro.

2. Materials and methods

2.1. Characterization of Mg and Mg–Ti microparticles using scanning electron microscopy (SEM)

Mg particles (Goodfellow, Product #: MG006021), generated by mechanical abrasion, with 99.8% Mg purity, were galvanically coupled with Ti (Alfa Aesar, Stock #: 13975 and Lot #: C19N26), with 99.99% Ti purity. These particles are formed from a mechanical process (e.g., cutting or milling). Magnesium particles are then sieved to the required particle range so that most of the particles have the maximum diameter of approximately 50 μm. Mg and Ti were galvanically coupled via direct current sputtering (Denton Systems) for 5 min, at 1.2 kV at 50 mA and 100–200 mTorr. Sputtering allowed the Ti layer to cover only half of the Mg microparticles (the top that was exposed during sputtering) due to the line-of-sight nature of the sputter process, while leaving the bottom half of the Mg microparticles uncovered. SEM images of Mg and Mg–Ti microparticles were taken in secondary emission mode. Scanning electron microscopy (SEM, JEOL 5600, Dearborn, MA) with energy dispersive spectrometry (EDS, Princeton Gamma Tech, Princeton, NJ) were also used to characterize the particles.

2.2. Measuring thickness of Ti layer using atomic force microscopy (AFM)

Due to the extremely thin layer of Ti developed during sputtering, the thickness deposited was investigated by covering half of silicon dioxide (glass) slides while the other half was left exposed during sputtering with Ti. Prior to sputtering, the glass surface was washed in soap and water, rinsed in distilled water, and rinsed in pure ethanol. The sputtering conditions remained the same, as mentioned above. AFM (Digital Instruments Nanoscope IIIa) was then used to take height and deflection images at the border in contact mode using a standard silicon nitride tip. AFM software was then used to measure the height and morphology of the sputtered layer.

2.3. Analysis of MC3T3 cell viability over Mg and Mg–Ti particle concentrations

Mouse fibroblast cells, MC3T3 (ATCC #: CRL-2593), were seeded in a 6-well plate ($A = 9.6\ cm^2$) with a cell density of 10,000 cells/cm^2, and were left for 12 h in the incubator at 37 °C and 5% CO_2 to allow cells to attach to the plate. The cells were cultured in a media made of minimum essential medium alpha medium AMEM (Cellgro Corning 15-012-CV), 10% fetal bovine serum FBS (Invitrogen 16000044), and 1% penicillin–streptomycin–glutamine PSG (Invitrogen 10378-016). Mg and Mg–Ti microparticle concentrations ranging from 50 to 1750 μg/ml were given to the cells for $t = 24$ h, with 2 ml of the solution given per well. The particles were randomly scattered, and so the local density of particles in different regions in the sample varied. Live/dead assay (Invitrogen #: L3224) was performed at the end of each time period to measure cell viability using an optical microscope (Leica Instruments). Ten images were taken per sample at random for three separate samples and the number of live and dead cells was counted manually in each image using Image J software (Image J, NIH Bethesda MD), using the manual cell counter feature. Manual counting of live and dead cells was most effective and accurate due to the software's inability to differentiate between cells and particles. Viability was determined as the number of live cells divided by the total number of cells in each image.

$$\%\mathrm{CV} = \frac{\#\text{of live cells}}{\#\text{of live cells} + \#\text{of dead cells}} \times 100$$