Differential magnesium implant corrosion coat formation and contribution to bone bonding

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Short title: Magnesium implant bone bonding

Abstract

Magnesium alloys are presently under investigation as promising biodegradable implant materials with osteoconductive properties. To study the molecular mechanisms involved, the potential contribution of soluble magnesium corrosion products to the stimulation of osteoblastic cell differentiation was examined. However, no evidence for the stimulation of osteoblast differentiation could be obtained when cultured mesenchymal precursor cells were differentiated in the presence of metallic magnesium or in cell culture medium containing elevated magnesium ion levels. Similarly, in soft tissue no bone induction by metallic magnesium or by the corrosion product magnesium hydroxide could be observed in a mouse model. Motivated by the comparatively rapid accumulation solid corrosion products physicochemical processes were examined as an alternative mechanism to explain the stimulation of bone growth by magnesium-based implants. During exposure to physiological solutions a structured corrosion coat formed on magnesium whereby the elements calcium and phosphate were enriched in the outermost layer which could play a role in the established biocompatible behavior of magnesium implants. When magnesium pins were inserted into avital bones, corrosion lead to increases in the pull out force, suggesting that the expanding corrosion layer was interlocking with the surrounding bone. Since mechanical stress is a well-established inducer of bone growth, volume increases caused by the rapid accumulation of corrosion products and the resulting force development could be a key mechanism and provide an explanation for the observed stimulatory effects of magnesium-based implants in hard tissue.

Keywords: Magnesium corrosion layer, osseointegration, pull out force, mechanical stress load, bone remodeling

1. Introduction

Magnesium alloys have promising properties for manufacturing the next generation of biodegradable medical implants including mechanical strength, light weight and the stimulation of bone growth[1-5]. In moist environments metallic magnesium reacts with water which results in the production of gaseous hydrogen (H_2), soluble magnesium ions (Mg^{2+}) and hydroxyl ions (OH^-). Rapid implant corrosion can lead to hydrogen accumulation and the formation of gas pockets in the tissue while the two oppositely charged ions can combine and precipitate to build up a protective corrosion layer on the implant that subsequently slows down the degradation rate[6]. In vitro corrosion in technical salt water corrosion solutions proceeded more rapidly than in vivo, which has been attributed to elevated concentrations of chloride ions and the solubility of magnesium chloride. In a biological environment, the incorporation of protective carbonates, calcium phosphates and proteins reinforces the magnesium corrosion layer which is thought to improve the biocompatibility of magnesium-based implants and to reduce side effects[7-10]. Calcium phosphate coatings are highly biocompatible and have also been proposed to promote new bone formation which would be beneficial for both, implant fixation and the healing of damaged bones[11].

Bone growth requires the differentiation of multipotential mesenchymal precursor cells, a process that is stimulated by secreted bone morphogenetic proteins (BMPs). BMPs can then bind to cognate receptors on responsive cells, leading to the phosphorylation and activation of Smad transcription factors and lineage-specific gene expression. In response, mesenchymal cells differentiate into various cell lineages such as cartilage-forming cells (chondrocytes), fat storage cells (adipocytes) or into bone-forming osteoblasts[12,13]. Among the differentiation-specific expressed proteins some have been used as specific markers for osteoblast activity, such as collagen type I that functions as a scaffold for calcium phosphate deposition, or the signaling protein osteocalcin[14]. Collagen Ia1 gene expression has been shown to be upregulated by Smad1 in cultured mesenchymal precursor cells during osteoblast differentiation while collagen IIa1 has been shown to be highly expressed in chondrocytes. Indeed, enhanced collagen Ia1 expression after osteogenic differentiation in the presence of excess magnesium ions or early increases in osteocalcin protein levels have been observed in bone cells in the vicinity of magnesium alloy implants[15,16]. Gene expression analysis can therefore be used to detect bone precursor cell differentiation and bone formation.

Based on histological data and from implant pull out forces, bone conductive properties of magnesium alloy implants were observed and proposed to be mediated by magnesium hydroxides as well as by calcium phosphates present in the magnesium corrosion layer[17-22].

However, the mechanism of bone growth stimulation by magnesium is not well understood and the efficacy of calcium phosphate coatings on implants to improve bone growth have been discussed controversially in clinical and animal studies[23,24]. Similarly, under particular conditions, elevated magnesium ion concentrations have been suggested to promote the differentiation or re-differentiation of cultured mesenchymal precursor cells[25,26].

Since so far an unequivocal mechanism of action could not be identified by using various cell culture models and in vivo studies, the present study was initiated to characterize mechanisms that could contribute to the enhanced osseointegration of magnesium-based implants. For structural reasons magnesium alloying allows for superior mechanical properties[27]. Nevertheless, throughout the present work pure metallic magnesium samples were consistently used to facilitate the interpretation of the results and to avoid complications by potentially cytotoxic alloy components such as rare earth elements[28]. The results of cell culture assays did not support stimulatory effects of excess Mg^{2+} ion concentrations on osteoblast precursor cell differentiation. Neither were cytotoxic effects of the pure corrosion product magnesium hydroxide compatible with the stimulation of bone growth. However, in an in vitro assay the accumulation of corrosion products lead to the exertion of force in hard tissue. Mechanical stress is a well-established bone stimulatory mechanism and could therefore explain the previously observed bone fortification near magnesium implants in vivo.

2. Materials and Methods

2.1 Mesenchymal precursor cell differentiation in the presence of magnesium additions

Recombinant bone morphogenetic protein 2 (rBMP2)-expressing C3H10T½ murine mesenchymal precursor cells (C3H10T½-BMP2) and native C3H10T½-cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Sigma D-7777 supplemented with 10 % fetal calf serum (Gibco), 6 mM glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin) and allowed to differentiate during incubation in differentiation medium (DMEM, 10 % fetal calf serum, 50 µg/ml ascorbic acid (Sigma) and 10 mM beta-glycerophosphate (Sigma) in the absence of exogenously added BMP2 protein), as previously described[29,30]. According to the manufacturer DMEM contains 0.81 mM $MgSO_4$. Various amounts freshly prepared 1 M $MgSO_4$ (Sigma) stock solution in deionized sterile water were added to the osteoblast differentiation medium to increase the final Mg^{2+} ion concentration from 1 mM up to 50 mM as indicated. For rBMP2 expressing cells 5 µg/ml puromycin (PAA Laboratories) were added to maintain rBMP2 expression during differentiation. Cells were incubated in a 12-well tissue culture plate in differentiation medium for 2 weeks in the presence of the indicated amounts

of magnesium granulate and were then stained with 0.5 % (w/v) Alcian blue 8GX (Sigma) dissolved in 0.1 N HCl. Alkaline phosphatase (APase) activity was detected by a staining reaction using SigmaFast™ Nitro Blue Tetrazolium/5-bromo-4-bhloro-3-indolyl phosphate ready-to-use tablets according to the manufacturer's recommendation (Sigma). Differentiation-specific gene expression analysis was performed as previously described in detail, including the primer nucleotide sequences[31]. Briefly, differentiated cells were collected; total RNA was extracted with the TriReagentLS according to the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH, U.S.A.). 5 µg total RNA were subjected each to reverse transcription polymerase chain reactions (RT-PCR) with primer pairs specific for the genes indicated. Depending on the gene, 20 to 30 PCR cycles were performed and the resulting DNA fragments were separated by agarose gel electrophoresis, visualized by ethidium bromide staining and photographed under UV illumination. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) served as a constitutively expressed reference gene.

2.2 Animal handling

Female Balb/c mice (Harlan Winkelmann, Borchen, Germany) were kept by professional animal care takers in individually aerated specific pathogen-free cages on a standard diet with water ad libitum. Magnesium (99.94% pure) discs were 5 mm in diameter and 2 mm in thickness[32]. The 0.05% deviation in the purity compared to the magnesium pins was considered to be acceptable and was due to the fact that the magnesium was obtained from two different sources. Magnesium hydroxide-containing implants were prepared by mixing crystalline magnesium hydroxide with deionized sterile water and by manually filling the resulting slush into the pores of titanium discs followed by drying at 37ºC for 1 week. Porous titanium disc implants 7 mm in diameter and 2 mm high were used as carriers for the mechanical support and to prevent an uncontrolled dissolution of magnesium hydroxide in the tissue. For comparison empty porous titanium discs (Ti) served as controls. The porous discs and surgical implant procedures have been depicted and described elsewhere in detail[33]. Briefly, the animals were anesthetized; a subcutaneous pocket was created on the lower back where the discs were inserted. The wounds were closed by an interrupted suture. Pictures were taken by using an in vivo imager (Xenogen IVIS 200, Perkin Elmer). For 3 disc shaped implants of each material type one animal was used for surgery, overall 3 animals were sacrificed. All animal experiments were conducted according to ethical guide lines of the

Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) under the permission 33.42502/07-10.5.

2.3 In vitro magnesium implant corrosion

Pure magnesium (99.99%) wires and titanium wires (both form Good fellow Cambridge Ltd., Huntingdon) with diameter of 0.4 mm were cut into 15 mm long pins. For pull out tests cleaned tibia bones derived from a total of 24 female Balb/c mice (Harlan-Winkelmann, Borchen, Germany) were sterilized by incubation in 4% formalin solution for 7 days at room temperature. The joint was cut off on one end of the bone and a magnesium pin was inserted 5 mm deep into the medullary cavity such that 10 mm was sticking out for fixation in the pull out device. For each assay groups of 3 magnesium pins and, for comparison, 3 corrosion-resistant titanium pins were inserted in the prepared murine tibia bones. Corrosion was initiated by incubation in cell culture corrosion medium DMEM was used containing 1% (v/v) glutamine and 10% (v/v) foetal calf serum at 37^{o}C in a humidified atmosphere containing 5% CO_2 in a cell culture incubator. Spent cell culture medium (supernatant) that was used as a corrosion medium was collected from confluent NIH3T3 (ATCC CRL-1658) mouse fibroblast cultures after 3 days of incubation. Corrosion was allowed to proceed for various time periods up to 30 days as indicated. After corrosion the bones containing magnesium implants were immobilized at the remaining joint in a metal fixture by using a three component cold-curing resin (Rencast FC52/53 Isocyanate, FC53 Polyol, Füller DT 082; Gössl & Pfaff GmbH, Karlskron/Braulach, Germany). The maximum uniaxial pull out force was determined with a materials testing device (Zwick/Roell 1445, Zwick GmbH & Co KG, Ulm, Germany) with no pre-load force at a velocity of 0.5 mm/min. For each experiment the average value of 3 independent assays and the standard deviation were calculated.

2.3 Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDX)

Magnesium samples were dried at 37°C for 3 days and then fixed on a sample holder with a conducting glue foil. Electron microscopic SEM and EDX analyses were performed at 10 KeV acceleration using a fully automated variable pressure microscope (REM-S3400N, Hitachi). Electron microscopic images were taken at a magnification of 200-fold with an emission current of 80-110 A and at a working distance of 21-22 mm. Elemental analyses were performed as previously described by using the standard ZAF method (Z, atomic number effect; A, absorption correction; F, fluorescence correction)[34]. For scanning electron microscopy with X-ray microanalysis (image-EDX) samples were imaged in a Zeiss Merlin

microscope at a magnification of 174 and a working distance of 8.4 mm; the acceleration voltage was 20 kV and the emission current 500 pA. For image- EDX the Oxford AZtec software version 1.2 was used. EDX was performed with a 50 square mm X-Max SDD-detector (Oxford Instruments) at a resolution of 512x384 pixels and 5 frames, a dwell time of 100 sec and with the process time set to 4. Quantification of the elemental distribution was done over the entire area depicted by excluding the carbon background with the standard method of the AZtec software version 1.2 (Oxford Instruments).

3 Results

3.1 Magnesium additions to cell cultures fail to stimulate osteogenic cell differentiation

Magnesium granules were added to cultures of differentiating mesenchymal precursor cells to investigate if soluble magnesium corrosion products were involved in the stimulation of bone growth. Recombinant bone morphogenetic proteins (rBMP)-2 expressing cells were used to stimulate the differentiation process. After two weeks differentiated cells were visualized (Fig. 1). In agreement with previous observations, cells did not survive in close contact with magnesium granules (Fig. 1; void areas in the cell culture wells)[25,35]. In addition, metallic magnesium did lead to decreased chondrocyte and osteoblast-specific staining intensity, respectively. Since cell death due to pH increases could have masked other effects, it was specifically investigated whether elevated magnesium ion concentrations could have differentiation-promoting effects. Differentiation-specific staining confirmed requirement for rBMP2 expression for osteoblast and adipocyte differentiation (Fig. 2). On the other hand, no noticeable effect on osteoblast or adipocyte differentiation was observed in response to increased magnesium ion concentrations up to 10 mM (Fig. 2; A, B2 Ost to B2 Adp). In line with a previous report chondrocyte-specific differentiation even decreased under these conditions (Fig. 2; A, B2 Cnd)[36]. Further increases in magnesium ion concentrations up to 50 mM lead to a reduction of osteoblast cell densities (Fig. 2; B, B2 Ost). While minor effects on differentiation-specific collagen expression were detected, the reduced osteoblast activity after differentiation in the presence of elevated magnesium ion levels was confirmed by the osteocalcin gene expression analysis with respect to a constitutively expressed house-keeping gene (Fig. 2; C, ColI and OC compared to HPRT). Consistent with data from human cells, in these experiments metallic magnesium or elevated magnesium ion concentrations lead to reduced cell density[37]. In summary, these straight forward cell culture experiments did not provide any evidence for the chemical stimulation of mesenchymal precursor cell

differentiation. Therefore, it was investigated whether other factors could contribute to the observed bone bonding of magnesium-based implants.

3.2 Magnesium hydroxide loaded implants act cytotoxic

To examine if magnesium hydroxide as a chemical substance could be sufficiently biocompatible to be considered as a candidate for the induction of bone bonding of magnesium implants in vivo, the effects after subcutaneous implantation in mice were investigated. Previously characterized porous titanium discs were used as a sturdy and biocompatible carrier to avoid fragmentation and premature solubilization the magnesium hydroxide. Whereas both, metallic magnesium and plain porous titanium implants appeared biocompatible, two magnesium hydroxide-containing implants caused tissue necrosis and a minor skin irritation resulted from a third implant (Fig. 3, posterior implants versus anterior implant). Even though magnesium corrosion results in a magnesium hydroxide-containing coating, there was a distinct difference in the tissue response between the biocompatible magnesium implant and the cytotoxic activity of crystalline magnesium hydroxide. Therefore, pure magnesium hydroxide did not appear to be a likely candidate that could be responsible for the osteoconductive properties of magnesium implants.

3.3 Corroding magnesium implants interlock with bone in the absence of viable cells

To investigate if corroding magnesium implants could bond to bone in the absence of cellular activity murine tibia bones were explanted and cells were inactivated by incubation in formalin. Then the joint was removed from one end of the bone and magnesium pins were inserted into the intramedullary cavity. To mimic in vivo corrosion conditions, the bones with magnesium implants were incubated in either cell culture medium or to allow for the accumulation of cellular products, in cell culture supernatant (Fig. 4; A). For comparison, a protein-free phosphate buffer was used. After corrosion, the implant pull out forces were determined. The pull out force of magnesium pins increased in the first week after incubation in corrosion media and remained almost constant thereafter for up to three weeks (Fig. 4; C). Statistically significant increases in the maximum pull out force of magnesium pins were similarly recorded and reached a maximum after incubation period of 7 days in any of the three corrosion media. The minor decrease in the required force and differences between the corrosion media used remained below significance levels in these assays. In contrast, no increases could be detected at any time point in the pull out force of titanium pins that were used as an established long-lasting implant material that does neither induce bone-growth in

vivo nor exhibit relevant short-term corrosion effects under physiological conditions. The results showed that in the absence of cellular activity magnesium corrosion could lead to implant bonding to the surrounding bone.

3.4 Formation of a differential calcium phosphate-containing corrosion layer

To investigate if a growing corrosion layer could be the reason for the increases in the pull out force of magnesium implants the surface was investigated in more detail using electron microscopy. Freshly prepared magnesium pins appeared comparatively smooth with dispersed small deposits (Fig. 5; A). The largest elemental fraction of the surface was magnesium with lesser amounts of oxygen and carbon apparently due to inadvertent corrosion during manufacturing or storage of the magnesium wires. Due to the irregular corrosion pattern differential characterization of the bright and dark zones could not be performed in this analysis (for mapping of the individual elements by imaging see below). After submersion of the pins in cell culture medium a corrosion layer was detected with a rough structure reminiscent of charred wood appeared that contained elevated amounts of the elements oxygen, carbon, calcium and phosphate (Fig. 5; B). This is consistent with previous reports and with the generation of the lowly soluble corrosion product magnesium hydroxide and possibly magnesium carbonates with protein and calcium phosphate additions from the cell culture medium. Freshly prepared cell culture medium may not optimally reflect the corrosion conditions in living tissue. To test if cellular products could possibly affect the corrosion assays were also performed in medium supernatant that was derived from mammalian cell cultures. The pH value was lower than that of fresh medium, an effect ascribed to acidic by-products of cellular metabolism such as lactate. This phenomenon is physiologically relevant and is known to occur during exercise in human tissue and it is also reflected in mammalian cell cultures, particularly at high cell densities[38,39]. While the elemental composition of the magnesium corrosion layer was similar, the corrosion process appeared to have progressed faster during incubation in cell culture supernatant and resulted in a coarser surface appearance (Fig. 5; C). This correlated with a more acidic pH of the cell culture medium supernatant that could be expected to accelerate magnesium corrosion (Table 1). On the other hand, a crystalline surface and as expected, the absence of calcium was observed after incubation in phosphate buffer (Fig. 5; D). In conclusion, during exposure to various physiological liquids the formation of corrosion layers on magnesium pins correlated with increases in the pull out force for implanted pins that were similarly treated.

To collect further evidence of physical interactions of the magnesium implant corrosion layer with bone the elements present on the pulled out pins were examined. As expected, the elemental analysis of the tibia bone revealed the presence of carbon, oxygen, calcium and phosphate (Fig. 6; A). Presumably due to the limited sensitivity of the technique nitrogen could not be detected. In accord with the notion of a bone interlocking corrosion layer the extracted pins appeared to be stripped from the corrosion layer and showed longitudinal scratches (Fig. 6; B to D). Compared to implanted magnesium pins exposed to cell culture medium, the phosphate buffer treatment appeared to lead to less degradation despite the acidic pH of the phosphate solution, which is consistent with the proposed protective role of magnesium phosphate coatings (Table 1)[40]. Interestingly, there was no calcium and phosphate detected on the surface of pulled out magnesium pins. The magnesium corrosion process could be expected to be slowed and altered within the bone, but since the corrosion layer appeared to be damaged after the pull out procedure, we speculated that these elements may have remained associated with the bone during the pull out procedure. In support of this notion, it has been reported that a calcium phosphate gradient exists in the corrosion layer[41]. To investigate if this could have been the case, magnesium corrosion layer compositions were examined in detail with a more sensitive assay. On untreated magnesium pins the element magnesium with occasional oxidation spots (Fig. 7; 0h and Table 2). The amount of calcium and phosphate was below detection levels and could not be visualized (Fig. 7; 0h, Ca and P). However, already after one hour incubation in cell culture medium both, calcium and phosphate were detected on the entire surface, indicating that these components were rapidly accumulating (Fig. 7; 1h). After incubation for one week the calcium and phosphate contents increased further (Fig. 7; 7d and Table 2). After pulling out implanted magnesium pins the distribution of magnesium, calcium and phosphate appeared patchy and the overall content of the latter two elements was reduced (Fig. 7; 7d p and Table 2). This finding strengthened the notion that the corrosion layer composition was similar after corrosion within the bone but it appeared interlocked with the surrounding bone and partially disrupted during the pull out procedure. Most interestingly, when the corrosion coat was intentionally scratched off from magnesium pins that were freely submerged in corrosion liquid, between the corrosion surface and the plain magnesium in the center of the pin a layer was exposed that contained reduced amounts of calcium and phosphate (Fig. 7; 7d s, white arrow). This was in agreement with a report showing that there was a concentration gradient for these elements as could be expected if the calcium phosphate were precipitating from the surrounding medium to the corrosion surface.

3. Discussion

The scientific literature contains apparently contradictory data concerning the properties of magnesium alloy implants such as the corrosion rate, biocompatibility, gas accumulation and osteoconductivity. However, these results depend on numerous, difficult to standardize sample manufacturing procedures, experimental set ups and assay conditions (Table 3). The corrosion rate plays a central role for the biological effects, yet it is non-linear and dependent on multiple effects at the metallic interface. Variable factors such as the exposure to water molecules, corrosive or protective ions from the environment and the dynamic formation of a protective corrosion layer play a key role in vitro and in vivo. The experiments in this study revealed that even minor changes in vitro under physiological conditions such as the inclusion of cellular products or the exposure to an increased carbon dioxide atmosphere can influence the pH and therewith the corrosion rate. Histological findings and increases in the pull out force of magnesium alloy implants have been taken as evidence for the stimulation of bone growth in experimental animals whereby the detailed mode of action remained to be defined. Even though cell culture experiments cannot reproduce the complexities of bone formation in vivo, this approach is frequently the first choice and has successfully been applied to elaborate numerous molecular details of bone formation. In agreement with previous reports, metallic magnesium or soluble magnesium corrosion products lead to reduced viability of cultured cells rather than to enhanced osteogenic cell differentiation[42,43]. In addition, after implantation of magnesium hydroxide-containing implants into soft-tissue, signs of necrosis were observed in our mouse model. These findings were in line with reports of cytotoxic effects of magnesium in vitro against mammalian cells and bacteria that appeared to be mainly due to pH increases[32,44,45]. Whereas pH increases are the result of magnesium corrosion and magnesium hydroxide production it is conceivable that in comparison to pure crystalline magnesium hydroxide additional components such as proteins or phosphates in metallic magnesium implant corrosion layers could enhance the biocompatibility in vivo[46,47]. The findings do not entirely exclude the possibility that under particular conditions magnesium corrosion, excess Mg^{2+} ions or moderate pH increases could stimulate the differentiation of osteogenic cells[48]. However, it was considered less likely that established cell differentiation procedures comprise suboptimal pH or Mg^{++} ion levels and therefore no further attempts were made to optimize these parameters. Instead, a fundamentally different approach was chosen that allowed for physical parameters that could not be investigated in cell cultures.

While useful for basic implant material characterization in soft tissue, the small dimensions of the mouse impede investigations of bone implants. For this reason an acellular in vitro approach was used to investigate physico-chemical bone-magnesium implant interactions. The mechanical consequences of implant volume increases due to the accumulation of corrosion products and the resulting forces in hard tissue were investigated. Increases in the pull out resistance of magnesium implants from avital bone suggested that the accumulation of corrosion products was sufficient to fill void spaces and interlock magnesium implants with the surrounding bone in the absence of living cells. Importantly, in this study evidence is presented that even in the absence of live cells, the geometry of the implantation site and the stiffness of the bony tissue permits the generation of force which may constitute an important factor in the observed stimulation of bone growth by corroding magnesium implants in vivo (Table 3). It appeared likely that rather than increased magnesium ion concentrations, increasing pressure could be a key parameter for the reported bone stimulatory effects of magnesium alloy implants in vivo. While in vivo both, the increasing volume of the corrosion layer and bone growth could contribute to the fixation of magnesium implants, the two parameters cannot retrospectively be distinguished by common histological analyses. Moreover, the timing may be of importance due to the transient period at which void spaces are occupied by corrosion products: At early time points the corrosion layer may not be fully developed while at later time points the dissolution of the corrosion products may exceed the production, leaving gaps that may hamper the interpretation of histological data. In addition, even though it may appear less likely, presently it cannot entirely be excluded that in a tightly closed bone cavity during magnesium corrosion hydrogen accumulation could also contribute to pressure increases and thereby stimulate bone growth. Based on the findings in this study a working model for the stimulation of bone growth by metallic magnesium implants could be derived. Thereby, in a first phase the accumulation of solid magnesium corrosion products would lead to the implant interlocking in the absence of osteogenic cell activity. Then, in a second phase, further corrosion product accumulation would increasingly exert pressure on the adjacent bone. It is an established fact that force applied to bony tissue stimulates bone fortification and bone growth (Wolff's law)[49]. In parallel, calcium phosphate and organic material incorporation at the corrosion coat surface would support osteogenesis by avoiding excessive pH increases caused by magnesium hydroxide and thereby provide a biocompatible and non-inflammatory interface, which is another prerequisite for bone growth.

The proposed mechanism implies additional consequences. Osteoconductive but no osteoinductive effects of magnesium implants have been reported and would be expected,

since pressure in response to implant volume increases could build up only if the implant is entirely enclosed in a limited space in hard tissue, but not in yielding soft tissue or at tissue interfaces. Therefore, minimal forces and minor bone induction if any would be predicted from subcutaneous magnesium implants or from implants in open cavities[50]. In addition, more slowly corroding implants such as zinc or iron alloy implants would be expected to have delayed or minor bone-stimulatory effects, if any[51,52]. In particular, iron lead to the accumulation of inflammatory, insoluble corrosion particles which is thought to be detrimental for bone formation.

Overall, the results suggested that under appropriate circumstances the accumulation of magnesium implant corrosion products could build up pressure when entrapped in hard tissue. For this to occur the corrosion must be rapid enough to at least temporarily permit the accumulation of corrosion products to fill any void space between bone and implant. We propose that this mechanism is the key for the documented stimulation of bone growth by magnesium alloy implants in vivo. However, whether more quickly degrading magnesium alloys would result in increased pressure and enhanced bone induction as predicted by the model remains to be investigated.

4. Conclusion

An in vitro bone-implant model was established to understand magnesium-bone interactions in the absence of cells. The increasing corrosion layer on magnesium implants was sufficient to exert force and interlock implants with the surrounding bone in the absence of bone growth. Transient pressure exerted by accumulating corrosion products could provide an explanation for the reported stimulatory effects of metallic magnesium-containing implants that are enclosed in bony tissue. In addition to a sufficiently rapid corrosion, the dimensions both, the implant and the implantation site, as well as the rigidity of the surrounding bone could constitute essential aspects in this process.

5. Acknowledgements

We thank Thomas Ebel from the Helmholtz-Zentrum Geesthacht for manufacturing and providing porous titanium disks. The project was funded by the German Research Foundation (DFG) collaborative research grant SFB 599 “Sustainable Bioresorbable and Permanent Implants of Metallic and Ceramic Materials”. M.I.R. was supported by a joint grant of the German Academic Exchange Service (DAAD), Germany, and the Higher Education Commission of Pakistan.

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Figure Legends

Figure 1. Reduced differentiation of cultured mesenchymal precursor cells to osteoblast in the presence of metallic magnesium. Recombinant bone morphogenetic protein 2 (rBMP2) expressing C3H10T½ mesenchymal precursor cells (B2) were allowed to differentiate for 2 weeks in a 12-well plate in the presence of the indicated amounts of metallic magnesium granulate (Mg). Chondrocytes were then stained with Alcian blue (B2 Cnd) and osteoblasts were assayed for alkaline phosphatase activity (B2 Ost) and photographed. Enlarged sections shown to the right are photomicrographs of cells taken with a light microscope. (-) designates control cultures without magnesium granulate additions. Experiments were performed in triplicates and representative pictures were selected.

Figure 2. Inhibitory effect of elevated magnesium ion concentrations on the differentiation of cultured mesenchymal precursor cells. (A) and (B), rBMP2 expressing C3H10T½ mesenchymal precursor cells were allowed to differentiate for two weeks in the presence of the indicated increased concentrations of magnesium ions (Mg^{2+}) that were added to the basal level of 0.81 mM $MgSO_4$ present in the original differentiation medium. Subsequently, osteoblast-like cells were assayed for alkaline phosphatase activity (B2 Ost), adipocytes were stained with oil red O (B2 Adp) and chondrocytes were stained with Alcian blue (B2 Cnd). As a negative control for differentiation-specific staining, original C3H10T½ cells were subjected to the same treatment in the absence of rBMP2 production and in the absence of exogenous BMP2 additions to the cell culture medium (Ost, Adp and Cnd, respectively). (C) Osteo-and chondrocyte-specific gene expression. rBMP2 expressing C3H10T½ cells were differentiated for 2 weeks in the presence of increased magnesium ions at the concentrations indicated (Mg^{2+}). Thereafter, cells were collected and total cellular RNA was extracted and subjected to reverse transcription polymerase chain reactions (RT-PCR) with primer pairs specific for the genes indicated. The resulting DNA fragments were separated by agarose gel electrophoresis and stained with ethidium bromide. The negative of the gel image is shown. Experiments were performed two times independently. PCR fragments are as follows: Col1, collagen type I, alpha 1; Col2, collagen type II, alpha 1; OC, osteocalcin; HPRT, Hypoxanthine-guanine phosphoribosyltransferase.

Figure 3. Magnesium hydroxide-containing implants lead to tissue necrosis. 3 Magnesium discs with a diameter of 5 mm (Mg), 3 crystalline magnesium hydroxide filled porous titanium discs with a diameter of 7 mm that were used as a carrier ($Mg(OH)_2$) or for comparison 3 empty porous titanium discs (Ti) that served as controls were subcutaneously implanted at the back of standard Balb/c mice. Pictures of the animals were taken individually immediately after the implantation procedure (d0) and then again one week later (d7).

Figure 4. Increases in the pull out resistance of corroding magnesium implants in the absence of live cells. 15 mm long magnesium pins with a diameter of 0.4 mm were inserted into the medullary cavity of sterile murine tibia bones and incubated in corrosion solutions at 37°C with 5% CO_2 (A). After incubation for the number of days indicated, the intact end of the bone and the end of the magnesium pin (arrow) were fixed in a force measuring device (B) and the pull out force was determined (C). Abbreviations are as follows: Med, pull out force of magnesium pins inserted tibia bones after incubation in freshly prepared cell culture medium; Sup, pull out force of magnesium pins after incubation in cell culture supernatant; KH_2PO_4, pull out force of magnesium pins after incubation in 100 mM KH_2PO_4 buffer; Ctr, pull out force of titanium pins incubated in fresh cell culture medium. Experiments were done in triplicates and the bars represent the average values of the maximum pull out forces with the standard deviation. Significant differences in the pullout forces were identified by an unpaired t-test, * indicates $p < 0.05$; *** indicates $p < 0.01$.

Figure 5. Magnesium pins develop a corrosion layer after incubation in cell culture media. Magnesium pins with 15 mm length and 0.4 mm diameter (A) were incubated in cell culture medium (B), or in cell culture medium supernatant of a three days old culture of fibroblasts at a cell density of 5×10^4 cells/ml (C) or in 100 mM KH_2PO_4 (D), respectively, for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. The pins were dried and imaged by scanning electron microscopy (images on the left side). The elemental compositions of the surfaces within the white squares were determined by EDX (shown on right side of each image). Peaks correspond to energies emitted by electrons returning to the K-shell. The scale bars in SEM images correspond to 100 µm.

Figure 6. Absence of calcium and phosphate on the magnesium implant surface after the pull out procedure. Magnesium pins inserted in bones and after determination of pull out force photographed using binocular microscope as described in the legend of Figure 4 (B-D). EDX spectrum of the internal cavity of a murine tibia bone (A), magnesium pins pulled out from a tibia bone after 1 week incubation in cell culture medium (B), in cell culture medium supernatant (C) and in 100 mM KH_2PO_4 (D), respectively. White rectangles indicate the region that was subjected to EDX analysis. EDX energy peaks correspond to emissions by electrons returning to the K-shell.

Figure 7. Decreased calcium and phosphate content below the magnesium corrosion surface exposed after mechanical shear force application. Magnesium pins with a diameter of 0.4 mm (0h) were incubated in standard DMEM cell culture medium in the presence of 10% fetal calf serum albumin (FCS) for the time period indicated. After corrosion the samples were blotted dry on a paper towel, air dried and subjected to reflection electron microscopic analysis (SEM and EDX). The elemental distribution on the exposed surface as indicated is indicated by the color intensity of the picture, the chemical symbols of the respective elements are given on top of the Figure. Three magnesium pins were inserted into the medullary cavity of an explanted murine tibia bone and after corrosion they were pulled out for analysis (7d p), one typical sample is shown. Three magnesium pins were corroded and then the corrosion layer was partially removed to expose the regions of the corrosion layer below the surface (7d s, white arrows).