Formation of an organic coat and release of corrosion microparticles from metallic magnesium implants

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Abstract

Magnesium alloys have been proposed as prospective degradable implant materials. To elucidate the complex interactions between the corroding implants and the tissue magnesium implants were analyzed in a mouse model and the response was compared to that induced by Ti and by the resorbable polymer polyglactin, respectively. One month after implantation distinct traces of corrosion were apparent but the magnesium implants were still intact, whereas resorbable polymeric wound suture implants were already fragmented. Analysis of magnesium implants two weeks after implantation by Energy-Dispersive X-ray spectroscopy (EDX) indicated that magnesium, oxygen, calcium and phosphate were present at the implant surface. One month after implantation the element composition of the outermost layer of the implant was indicative of tissue without detectable levels of magnesium, indicating a protective barrier function of this organic layer. In agreement with this notion, gene expression patterns in the surrounding tissue were highly similar for all implant materials investigated. However, high-resolution imaging using energy filtered transmission electron microscopy (EF-TEM) revealed magnesium-containing microparticles in the tissue in the proximity of the implant. The release of such corrosion particles may contribute to the accumulation of calcium phosphate in the nearby tissue and to bone conductive activities of magnesium implants.

Key words: Animal model; Biocompatibility; Biodegradation; Bone repair; In vivo test; Magnesium; Metal ion release; Osteoconduction

Abbreviated Title: Magnesium implant-tissue interactions

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Introduction

The body responds to foreign implant materials by a conserved series of events including the almost immediate formation of a proteinaceous layer on the implant surface. Within the first hour, immune cells are attracted from the blood, most rapidly neutrophils that are later followed by monocyte-derived macrophages. While the neutrophil accumulation is transient only, the macrophages remain in the granulation tissue and govern the subsequent inflammatory and wound healing responses such as the formation of a fibrous capsule [1]. Implants that are no longer required are commonly removed after the healing process is completed. For temporary applications degradable medical implants could overcome the requirement for revision surgery [2]. With respect to resorbable polymers, corrodible metal alloys appear to be less inflammatory and have superior mechanical properties in bone repair or as vascular stents [3-6]. Magnesium alloys are presently investigated as promising biodegradable implant materials and could circumvent major side effects of current stents [7]. In bone repair magnesium-based implants could help to reduce bone loss due to stress shielding effects that are associated with current permanent materials [4, 8-10]. In addition, there are indications that magnesium acts osteoconductive and could promote bone repair [4, 5, 11-13]. Magnesium has even been shown to act antibacterial and could therefore antagonize difficult to treat implant-associated infections [14-16]. After implantation magnesium corrodes by reacting with water whereby magnesium ions, hydrogen and hydroxide ions are generated [17, 18]. These products have diverse biological properties. Cells contain up to 20 mM of the magnesium ions and excess amounts can be excreted [19-23]. However, rapid and irregular corrosion and excess hydrogen production could lead to premature mechanical implant failure and to gas-filled cavities in the tissue,

respectively [24]. If the degradation rate exceeds the buffering capacity of the tissue, hydroxide ions could lead to pH increases and cell death at implant-tissue interfaces [25]. Therefore, various strategies have been devised to improve the corrosion resistance, such as anti-corrosive implant coatings, optimization of alloy compositions or manufacturing processes such as for the generation of metallic glasses [14, 26-35]. Such novel materials are conventionally first tested in vitro. Due to still incompletely understood interactions between corroding implants and the surrounding tissue, no standard in vitro testing protocols have been established that could reliably predict the behavior of degradable implant materials in vivo [26, 36-38]. Consequently, animal models are essential to validate the in vitro results. Mouse models have specific advantages for evaluating tissue-implant material interactions, such as the availability of genetically highly defined mouse strains and strains that mimic human disease states that facilitate the generation of reproducible results and the elucidation of molecular mechanisms [39-44].

In this study tissue interactions with magnesium implants were evaluated in a simple mouse model in a side-by-side comparison with two diverse clinically established materials, titanium and the resorbable wound suture material polyglactin [45, 46]. Histology and gene expression analyses were used to characterize implant-tissue interactions. Energy-Dispersive X-ray spectroscopy (EDX) and energy-filtered transmission electron microscopy (EF-TEM) were used to analyze implant surfaces and the adjacent tissue, respectively. In agreement with previous findings, corroding magnesium implants induced the deposition of calcium phosphates in the corrosion layer [14, 47-50]. Interestingly, magnesium-rich microparticles were detected in the tissue adjacent to magnesium implants.

Materials and Methods

Implant preparation

Wires of pure magnesium (99,9% magnesium, Good fellow Cambridge Ltd, Huntingdon, Great Britain) of 0.4 mm diameter were cut to pieces of 10 mm and incubated overnight in a aqueous solution of 1 M NaOH at 37°C to obtain a partially protective magnesium hydroxide layer [51]. The pieces were briefly blotted dry on filter paper, and then rinsed in sterile distilled water. The excess liquid was again removed with a filter paper and the samples were air dried and stored dry until implantation. Titanium wire (ARA-T Advance GmbH, Dinslaken, Germany) of 0.4 mm diameter was cleaned with ethanol and cut to 10 mm long pieces. Similarly, a 45 cm long, 0.4 mm in diameter, sterile resorbable surgical suture material Ethicon 6-0 coated Vicryl V489 (polyglactin 9.0) monofilament, a poly(glycolide lactide) copolymer (Aesculap Inc., Center Valley, PA) was cut to 10 mm long pieces for implantation into the mouse tails.

Animal handling

A total of 42 6-10 weeks old female Balb/C mice (Harlan-Winkelmann, Borchen, Germany) were kept under specific pathogen-free conditions in groups with a maximum of 5 animals per cage with individual aeration each. All animals were fed with a standard diet without lipid or cholesterol supplements and with drinking water *ad libitum*. Animals were anesthetized by intraperitoneal injection of ketamine (10mg/kg) and xylazine (4mg/kg). 10 minutes later the mice were laid on their back in a laminar flow hood and the mouse tails were disinfected with kodan (Schülke und Mayr, Norderstedt, Germany).The skin of the tail was pierced by a hypodermic

needle (18 gauge) and then a piece of magnesium wire, titanium wire or polyglactin monofilament was inserted into the lower tail artery as previously described except that no catheter was used [52]. The implantation site, the appearance and the behavior of the animals were visually inspected daily in the first week after implantation and later at least twice a week (Fig. 1). For gene expression analysis 36 mice were used in total (see below). 6 mice in total were used for histology, each with either consecutive 3 titanium, magnesium or polyglactin implants, respectively. Implants were examined 2 weeks and 4 weeks after implantation. From 3 animals each the two proximal 2 implants were used for histological analysis and the implant that was located towards the distal tail tip was used for electron microscopic analysis. The experiments were approved by the local authorities (permission nr. 33.42502/07-10.05).

Scanning Reflection Electron Microscopic (REM) analysis

The samples were air dried and then fixed on the support with a electrically conducting glue foil and analyzed using a fully automated variable pressure Hitachi REM S-3400N with Energy-Dispersive X-ray spectroscopy (EDX). The acceleration voltage used was 10keV, the distance was adjusted to 10 mm. Images were taken at a magnification of 200-fold at a working distance of 21 to 22 mm with an emission current of 80 to 110 µA. The quantifications were corrected according to the standard-less ZAF method (Z, atomic number effect; A, absorption correction and F, fluorescence correction). The energy peaks for the elements indicated correspond to the respective electron transitions to the K-shell, with the exception of titanium where energies corresponding to electron transitions to both, L and K-shell energies were

detected. The element composition of polyglactin implants could not be determined due to the low of electrical conductivity of the polymer.

Electron spectroscopic analysis

Segments of the mouse tail containing implants were isolated and the tail bone removed. The tissue was fixed in buffered glutaraldehyde solution (2.5% glutaraldehyde, 50 mM cacodylate, pH 7.4; 50 mM KCl, 2.5 mM $CaCl_2$) for 72 hours at 4 ºC. The samples were then washed for 30 minutes at ambient temperature in 20 mM HEPES buffer, pH 7.2 and subsequently dehydrated on ice in a series of increasing ethanol concentrations (10%, 30%, 50%, 70%, 90% ethanol in water, and then 100% ethanol, for 30 minutes each step) and eventually in pure acetone. The dehydrated tissue samples were stepwise infiltrated with epoxy resin monomer at room temperature (with one part acetone and one part resin for one hour; then with pure resin for one hour, followed by pure resin over night; and finally with pure resin for 6 hours) and then polymerized by flat embedment at 70 ºC for 15 hours. The embedded samples were cut to 70 nm thin sections and placed on Butvar coated 300 mesh hexagonal copper grids and analyzed with an integrated energy-filter transmission electron microscope (Libra 120 plus, Zeiss, Oberkochen, Germany) as previously described in detail [53]. Images were acquired in the elastic bright-field mode (zero-loss images) with an objective aperture of 60 µm and the energy-slit set to 10 eV width with a cooled 2k x 2k bottom-mount CCD (Sharp:Eye; Tröndle, Moorenweiss, Germany) at 120 kV acceleration voltage and an emission current of 1.0 to 1.5 µA. Three analytical electron spectroscopic methods were used for spectroscopic data acquisition; parallel electron energy-loss spectroscopy (PEELS), wide range PEELS (WR-PEELS) and electron spectroscopic imaging (ESI). 40 nm

ultrathin sections were picked up with 300 mesh hexagonal bare copper-grids without post staining. PEELS (Mg-K: 1190 – 1420 eV; Ti-L2,3: 350 – 577 eV) and WR-PEELS (Mg-K; Ti-L2,3: 46 – 1435 eV) were recorded at 120 kV and ≤ 1 µA emission current with acquisition times from 5 to 50 seconds/frame with 3 to 5 cycles. Original spectra were background corrected by the 'potence law' method and elemental maps of magnesium were calculated with the '3 window method', according to the image-analysis software (ESI-Vision, OSIS, Münster, Germany). For each electron-dense object analyzed the position within the tissue slice was carefully confirmed to exclude potential artifacts by superficial deposits.

Histological examination

Implants were removed after 2 weeks and after 4 weeks, respectively, and first examined using a Stemi SV11 stereo-microscope (Carl Zeiss Microscopy GmbH, Germany) equipped with an electronic camera Sony DSC S75 (see Fig. 2). Tail sections with or without implants were fixed in cacodylate-buffered 3.5 % formaldehyde solution, then dehydrated in a series of increasing concentrations of ethanol (10%, 30%, 50%, 70%, 90%, 100% ethanol in deionized water) and embedded in Technovit 9100 according to standard procedures [54]. To facilitate the subsequent thin sectioning, titanium implants were removed after fixation and prior to embedding while the softer magnesium and polyglactin implants were left in situ during the entire procedure. Tissue thin sections were stained with hematoxylin and eosin and then evaluated using an Axio Observer.A1 microscope (Carl Zeiss Microscopy GmbH, Germany; Table 1).

Gene expression profiling

Mice were sacrificed by cervical dislocation two weeks or four weeks after implantation, respectively. For each array analysis tail arteries of five mice together with three consecutive implants and the implant-associated fibrotic capsules were surgically isolated and shock frozen in liquid nitrogen. Tissue samples from tail arteries from 6 mice without implants were combined and used as a control. For RNA preparation the tissue samples were ground to powder in the presence of liquid nitrogen with mortar and pestle. Then RLT lysis buffer was added (RNeasy Fibrous Tissue Mini Kit, Qiagen, Hilden, Germany) and allowed to thaw while continuing to grind. Protein was hydrolyzed with proteinase K for 10 min. at 55°C and the RNA was purified as recommended by the manufacturer's protocol. RNA quality was examined using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). For biotin-labeled target synthesis starting from 3 µg of total RNA, reactions were performed using standard protocols supplied by the manufacturer (Affymetrix; Santa Clara, CA). Briefly, 3 µg total RNA was converted to double-stranded DNA using 100 pmol of a T7T23V primer (Eurogentec; Seraing, Belgium) that encoded a T7 promoter. The cDNA was then used directly in a T7 polymerase-dependent in-vitro transcription reaction in the presence of biotinylated nucleotides. The concentration of biotin-labeled cRNA was calculated based on the UV absorbance. 12.5 µg of each biotinylated cRNA preparation was fragmented and added to a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. The nucleic acid solutions were hybridized to MOE430 2.0 Affymetrix whole genome array GeneChips for 16 hours. Arrays were made in duplicates for each of the three implant materials. After hybridization the GeneChips were washed, stained with streptavidin-phycoerythrin and read out with an Affymetrix GeneChip fluidic station and scanner

using the standard Affymetrix GCOS 1.2 software using the default settings. For normalization all array experiments were scaled to a target intensity of 150. Analysis was performed using Array Assist 4.0 (Stratagene, Heidelberg, Germany). Genes were considered to be induced when the corresponding Affymetrix Probe set ID yielded a signal that was at least two-fold higher in the analysis of tail tissue extracts with implants with respect to the signal obtained from tail tissue without implants and, additionally, the signal was significantly above background levels (Present Call, $p \geq 0.01$) in at least one of the samples investigated. Regulated processes were identified by analyzing the induced genes using the computer program DAVID [55]. The number of genes indicated in Table 2 and Table 3 corresponds to the number of unique DAVID IDs that were identified for each biological process by the program. In a previously published gene array expression study mouse tail tissue with iron implants with 9 month implant residence time has been examined [52]. The iron implant gene expression data was re-calculated using the same parameters and programs described above and the result was included here to facilitate the comparison of the results of the previous study of iron implants with the implant materials investigated in this study (Table 2, Fe).

Results

Magnesium implant preparation and implantation procedure

To evaluate material-tissue magnesium interactions magnesium wires were inserted into the ventral tail blood vessel of mice. Even though magnesium alloys are preferred due to superior mechanical properties, pure magnesium was employed for this investigation to facilitate the interpretation of the results. As reference materials

the absorbable wound suture material polyglactin and pure titanium wires of the same diameter were implanted. This technique resulted in a precise and highly reproducible implant placement with minimal tissue injury but it was not specifically intended for testing vascular stent materials since the implant appeared to completely block the blood flow [52]. Initial attempts resulted in losses of up to 20% of all magnesium implants within the first few days after implantation. A few implants were observed that were partially protruding from the insertion site, suggesting that the losses could be due to implant extrusion. Since this phenomenon did not occur at later time points or with the other two implant materials, it was suspected to be caused by an initial burst in the generation of hydrogen gas [56]. Indeed, a thin, pre-formed corrosion layer that was not apparent to the naked eye completely prevented the premature magnesium implant losses [57]. Thereafter, all magnesium implants used in this study were passivated before implantation. Overall, the implantation was uncomplicated and no further adverse events were noticed with any of the three implant materials during the entire experimental period (Figure 1).

Moderate magnesium implant corrosion and surface layer formation in the mouse tail tissue

After residing 2 or 4 weeks in the tissue the implants were removed and examined by light microscopy (Figure 2, A to I). The regular metallic surface appearance of the magnesium wires turned uneven and splotchy with whitish to red-brown hues, indicative of organic material (Figure 2, A and B). 4 weeks after implantation the magnesium implants were still intact (Figure 2, C). Similarly, a thin, reddish-brown layer that in part peeled off had formed on titanium (Figure 2, D to F). Polyglactin implants appeared discolored and fragmented 4 weeks after implantation (Figure 2,

G to I). A more detailed examination of unstained implants by scanning reflection electron microscopy showed a cracked and pitted corrosion layer on magnesium implants after implantation (Figure 2; J to L). Since the samples were examined under vacuum it is feasible that the cracks resulted from the dehydration of the corrosion layer [56]. In agreement with the light microscopic analysis, after implantation magnesium implants were covered with a corrosion layer and in part, by an additional, apparently loosely attached surface layer that was also detected on the titanium samples (Figure 2, J to O). In conclusion, after 4 weeks in the mouse tail, the magnesium wire appeared moderately corroded and remained unbroken while the clinically established resorbable wound suture polymer turned out to be fragmented.

Magnesium implant corrosion zones containing magnesium and calcium phosphate deposits together with increasing amounts of organic molecules

To examine the element composition of the corrosion layer, implants were removed from the mouse tail and then subjected to EDX analysis. Before implantation the surface of the magnesium wire contained mainly magnesium, some oxygen and a lesser amount of carbon (Figure 3, A). Together with the surface appearance, this would be consistent with the presence of a minor MgO or $Mg(OH)_2$-containing corrosion layer. Most likely their formation may have resulted from the treatment with NaOH before implantation. It cannot be excluded that the reaction of magnesium with atmospheric oxygen and humidity could have contributed. The carbon could have originated either from reactions of magnesium with carbon dioxide in the air or alternatively, it could have been due to the manufacturing process. Two weeks after implantation the magnesium content at the surface was reduced. Instead, more carbon, oxygen and nitrogen were detected, which were most likely due to the

presence of organic material on the corrosion layer surface (Figure 3B). Despite the soft tissue environment and in addition to a minor amount of sodium, both, phosphate and calcium were present in the corrosion layer (see discussion). Four weeks after implantation the magnesium hydroxide content was below the detection limit, suggesting that the entire surface was coated by an organic layer (Figure 3C). In addition to titanium, oxygen and carbon were detected on titanium wires before implantation (Figure 3D). This was consistent with the presence of an oxide layer and of carbon remnants possibly originating from the manufacturing process. Similar to the magnesium implant, after two weeks the titanium peak was considerably reduced and after four weeks it was below detection limit. The distribution of the elements oxygen, nitrogen and carbon was very similar to that of tissue, indicative of a protein layer that concealed the metal and the oxide layer underneath (Figure 3, E to G).

Magnesium, calcium and phosphate-enriched ultrastructures in the vicinity of magnesium implants identified by EF-TEM

Energy filtered transmission electron microscopy (EF-TEM) was used to examine chemical elements present in ultrastructures in the tissue. This technique requires ultrathin sections that are prone to specific preparation artifacts that were carefully excluded from the subsequent analyses. (Figure 4; A to C). Screening of unstained tissue samples for magnesium within a distance of 20 to 40 µm to magnesium implants revealed clusters of small, up to 1 µm long electron dense structures with elevated magnesium concentrations (Figure 4, D). These structures contain magnesium precipitates as indicated by the PEELS analysis showing a distinct magnesium K-shell edge above the background (Figure 4E). This was confirmed by high-resolution ESI element mapping (Figure 4 E, insert). In addition, amorphous

structures with moderately elevated magnesium levels were detected (Figure 4, F). In one structure phosphate and calcium were detected by WR-PEELS. The 'phosphate-fingerprint' was typical for hydroxyapatite (Figure 4G). As expected, no increases of metal ions were detected near titanium or polyglactin implants (Figure 4 H and I). Due to their organic nature the polyglactin degradation products could not be distinguished from the tissue with this method. The nitrogen K-edge intensity peak was characteristic for proteins as the main cellular nitrogen-containing compound. In conclusion, microstructures in the tissue near magnesium implants were detected whose element composition corresponded to the composition of the corrosion layer, suggesting that microaggregates could be released from magnesium corrosion layers.

Similar tissue reactions to diverse implant materials

To assess the tissue compatibility of magnesium in more detail histological thin sections of mouse tails with diverse implants were compared (Figure 5). All materials tested appeared biocompatible with typical wound healing responses such as moderate amounts of granulomatous tissue surrounded by a fibrous capsule that was in part overlapping with the granular zone. In the tissue containing magnesium implants no evidence of tissue necrosis or inflammatory giant cells and no gas bubbles could be detected at any time (Figure 5, A and B). However, an acellular layer (remnants) apparently stripped of the implants was visible at the tissue-implant interface of both magnesium and titanium implants (Figure 5; B and F). The tissue responses were largely independent of the material and characterized by granulomatosis and to a lesser degree by a purulent appearance (Table 1). In addition, minor bleedings, indicated by the presence of red blood cells located in the

tissue outside of blood vessels, could be detected near all three types of implants. These inflammatory reactions did not increase further from two to four weeks implant residence time, whereas the size of the fibrous layer consistently increased from two to four weeks (Table 1). Even though the formation of a fibrous capsule can be considered as a standard tissue response to implants, the moderate and surprisingly similar response to all materials investigated indicated that under the conditions used the pure magnesium implants essentially behaved as tissue-compatible as the two conventional implant materials.

Wound healing identified as the most significantly regulated biological process independent of the implant material

For the molecular analysis of implant-tissue interactions a global gene expression analysis was performed. Briefly, one month after implantation RNA from tail tissue was prepared and subjected to gene array analysis. Most interestingly, the results showed highly similar patterns of gene expression in response to any of the implants materials investigated. The large majority of the genes that were more highly expressed in tissue with magnesium implants with respect to undisturbed tissue without any implants were also activated by polyglactin and titanium implants (Table 2, Total of Mg versus Ti, Gl, Mg). Most significantly genes involved in inflammation and wound healing processes were increased, including cytokine production and chemotaxis (Table 2). In a previous study long-term iron implants in the mouse tail were associated with the gene expression related to wound-healing processes[52]. To facilitate the comparison, the array data from previously analyzed iron implants were recalculated and the result included in this study (Table 2, Fe). Interestingly, the

same biological processes were activated by all four diverse implant materials investigated in two independent studies.

Genes that were expressed at a higher level in the tail artery tissue without implants were in biological processes related to muscle, metabolism, regulation of blood pressure and smooth muscle contraction (Table 3). This could indicate an implant-mediated loss of vascular tissue and its replacement by wound-healing associated fibrous tissue and inflammatory cell infiltrates.

Discussion

It is presently thought that after implantation the wounding and the presence of the foreign material lead to the recruitment and activation of monocytes that then govern the local inflammatory response and also coordinate the wound repair and foreign body response [58, 59]. It is assumed here that the implants clogged the tail blood vessel and presumably lead to the destruction of the vessel. For this reason this mouse model was intended to examine magnesium-soft tissue-interactions and not to serve as a model for vascular stents. To repair the damaged tissue the healing responses involve the activation of fibroblasts to proliferate, secrete collagen and other extracellular matrix proteins. Together they form a fibrous capsule and wall off the foreign implant material [60-62]. In agreement with these series of events, in response to all implant materials tested the histological evaluation revealed moderate, persistent inflammatory reactions and ongoing fibrous tissue formation during the first weeks after implantation. The side-by-side testing of magnesium with titanium and polyglactin allowed a comparison with clinically established materials of the durability of the implants and of the extent of inflammatory reactions. The

moderate responses to magnesium and titanium implants are in agreement with previous reports [63-65].. Interestingly, despite its clinically established excellent biocompatibility, titanium implants in the mouse tail tissue appeared to stimulate fibrosis even somewhat more potently than the two other materials.

Electron microscopic analyses of magnesium implants confirmed the uneven type of pitting corrosion which has been associated with premature material failure. In comparison to polyglactin the degradation of magnesium implants appeared moderate and this phenomenon was not further investigated, but various strategies to reduce the corrosion have been proposed elsewhere, such as optimized magnesium alloys, manufacturing processes or surface coatings [66-68]. Pure magnesium has a much lower Young Modulus value than pure titanium used in this study. Due to the actively moving tail of the mouse, stress corrosion could make magnesium more susceptible to crack formation in the protective layer of the implant. It remains to be examined if the corrosion rate could be even lower at alternative, potentially less stressful implant locations.

No magnesium on the surface of magnesium implants could be detected by EDX analysis 4 weeks after implantation. Even though the sensitivity of EDX is limited, these results can be taken as evidence that the magnesium implant is covered by an apparently acellular organic layer with little or no solid magnesium corrosion products at the surface. Elements detected in the outermost layer of the corroding magnesium implant 4 weeks after implantation suggested that the major component were proteins, which may have contributed to the moderate corrosion progression [69, 70]. In support of this notion it has been shown that in biological liquids a passivating corrosion layer forms around magnesium implants [71, 72].

While the corrosion layer dissolves, soluble magnesium ions are released and are expected to lead to an increased magnesium concentration in the implant surroundings. Gene expression was examined to detect whether in vivo the cellular response to magnesium implants could be influenced specifically by metallic magnesium or by its corrosion products. In accordance with the histological findings, magnesium implants did induce similar genes as the two clinically established materials. All materials investigated stimulated the expression of genes involved in wound-healing and in the foreign body response, indicating that at least during the first weeks after implantation these reactions dominate over material-specific responses. Titanium is stiffest material of the three materials tested in this and the somewhat more intense tissue reactions to titanium could be explained by implant micro-movements or mechanical stress to the cells rather than by chemical interactions [73-75]. Overall, the responses to magnesium detected by genomic gene expression analysis were largely independent of the material used and no specific responses to magnesium could be detected.

Magnesium has been reported to have bone-conducting activities; however, it is an unsolved question how the magnesium implants could stimulate bone growth or the accumulation of calcium phosphates in vitro or in bony tissue [11, 76-79]. Based on in vitro results it has been proposed that elevated levels of magnesium ions could stimulate bone or cartilage forming cells [80-82]. An alternative mechanism would be dystrophic calcification, a process whereby tissue destruction induces ossification ectopically in soft tissue [83]. However; in this study and additionally in a long-term investigation over 9 month no bone-forming cells and no extensive necrotic areas were apparent in the soft tissue near magnesium implants [84]. Similarly, there were no histological indications of initiating bone formation and gene expression profiling did not reveal any significant bone-specific gene expression. To our knowledge, the

magnesium accumulation in the tissue has not previously been examined with non-destructive methods at a resolution and sensitivity comparable to EF-TEM. The analysis revealed micrometer-size magnesium-rich deposits near magnesium implants. The element composition was in agreement with the elements that were identified in the magnesium corrosion layer, suggesting that the particles were most likely released from the implant corrosion layer. Such a process could be driven by disruptions of the corrosion layer by the continuous generation of magnesium hydroxide and hydrogen gas. Alternatively, increased levels of soluble magnesium ions in the surrounding tissue could induce the formation of microprecipitates. The accumulation of calcium phosphate in the magnesium corrosion layer appeared to occur independently of cellular activities. Calcium phosphates have been implicated in the stimulation of bone formation [85-87]. However, it is not clear how exactly the corrosion layer on the implant could influence bone-forming cells at a distance. In addition to increased concentrations of soluble magnesium ions near magnesium implants it is conceivable that corrosion particles that are dispersed in the tissue could contribute to the bone-stimulating effects of magnesium implants in bony tissue [4, 5, 11-13, 88]. This process is likely to be influenced by multiple factors and further work will be required to reveal the detailed mechanism of bony tissue responses to magnesium-based implants.

Conclusion

A mouse model that required minimal surgery in combination with simple implant forms was evaluated to investigate the basic material interactions of magnesium with soft tissue. It yielded a basic but detailed biological evaluation indicating similar tissue responses to pure magnesium implants in the mouse tail and to clinically

established materials. In view of the apparent barrier function of an organic implant layer that formed on the implants, the induction of calcium phosphate deposits and the release of corrosion microparticles into the surrounding tissue may contribute to the bone conductive effects of magnesium implants.

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Figures

Figure 1. **Minor wounding and inconspicuous inflammatory reactions to magnesium implants in the mouse tail.** Three magnesium pins with a diameter of 0.4 mm were inserted consecutively every 2 cm (demarcated with a felt pen) in the ventral tail vessel of Balb/c mice. The mice were anesthetized at the times indicated. Pictures were taken of the implantation site as follows: Tail with magnesium implants immediately after implantation (A); 5 days after implantation (B) and 10 days after implantation (C), respectively.

Figure 2. **Moderate but irregular corrosion of pure magnesium implants in the mouse tail.** Optical (A to I) and scanning reflection electron microscopic examination (J to L) of a magnesium wire (A to C; J to L), titanium wire (D to F, M to O) and glyconate filament (G to I) before implantation (A, D, G, J, M), two weeks (B, E, H, K, N) and four weeks after implantation into a mouse tail (C, F, I, L, O), respectively. The size bars shown on the lower right are valid for all light microscopic (A to I) and electron microscopic pictures (J to O), respectively.

Figure 3. Organic material, magnesium and calcium phosphate incorporation in magnesium implant corrosion layers. Magnesium (A, B, C) and titanium (D, E, F) wires before implantation (A, D), after 2 weeks (B, E) or 4 weeks (C, F) residence time in the mouse tail were analyzed by Energy-Dispersive X-ray spectroscopy (EDX) and Reflection Electron Microscopy (REM) (inset). Tail tissue without an implant was used as a control (G). The elements corresponding to the emitted x-ray energy are indicated above the respective peaks. The proportion of each element is indicated in the inserted tables. C, carbon; Ca, calcium; Mg, magnesium; Na, sodium; O, oxygen; Ti, titanium.

Figure 4. Magnesium-enriched microparticles in tissue adjacent to magnesium implants detected by Energy-filter transmission electron microscopy (EF-TEM). EF-TEM survey view of tail tissue adjacent to magnesium (A), titanium (B) and polyglactin (C) implants, respectively. Implants were removed prior to embedding and thin sectioning of the tissue samples, thereby leaving a void area in the image (v). The hexagonal black background pattern corresponds to the specimen support grid.

Preparation artifacts indicated are labeled as follows: Holes (h), partially overlapping tissue from section folds (f) and precipitates (circles) located on top of the section. Electron spectroscopic imaging (ESI) analysis of tissue adjacent to a magnesium implant (D). Magnesium-map (green), superimposed on the inelastic bright-field image of cellular ultrastructure (grey). Parallel electron energy-loss spectroscopy (PEELS) element analysis and ESI map (inset) of structural details in tissue adjacent to a magnesium implant (E), showing the energy-selecting slit position and its energy width (the part of the curve within the area shaded in green). The edge onset of the K electrons of magnesium in the spectrum derived from the electron-dense fragment that is encircled in the inset, is shown in the graph (Mg-K). PEELS analysis of electron dense patches from Mg (E, F) and Ti implants (H) as shown. The edge-onset corresponding to the Mg-K shell is indicated (Mg K), according to the Mg-reference spectrum of a MgO film, which is shown as a dark gray dashed line. Electron dense matrix cluster (inset) analyzed by WR-PEELS (G). The positions of typical hydroxyapatite relevant edges of P, Ca, and O (the P-L23 electron energy-loss near-edge fine structure (ELNES) together with a Ca-L23 and O-K energy edge) are indicated. PEELS from electron dense patches shown in the insets (1, 2, 3*)* of Ti implants and their corresponding spectra (H). The positions of the peak onset energies for nitrogen (N-K), titanium (Ti-L23) and oxygen (O-K) are indicated in the spectra. Measuring apertures and corresponding PEELS are adequately numbered. No EELS edges could be detected at energies losses corresponding to Ti-L23. Ti-L23 reference spectrum (dark gray dashed line). Inset scale bars are 0,5 µm. WR-PEELS analysis of two different areas of tissue adjacent to a polyglactin implant (I), and their measuring areas are numbered adequately. Ionization edges of Mg-K, and Ti-L23 are indicated and the corresponding reference spectrum is shown as a dark gray

dashed line. (Spectrum 2 is from tissue at a distance from the implant/ void area containing solely the embedding polymer as a resin control).

Figure 5. **Similar histological appearance of tissue responses to highly diverse types of implant materials.** Light microscopic pictures taken of histological sections of formaldehyde fixed, Technovit embedded and hematoxylin and eosin (HE)-stained mouse tail tissue 4 weeks after implantation. Prior to thin sectioning magnesium and titanium implants were removed while the polyglactin filament remained in situ. Mouse tail tissue after removal of a magnesium implant (A and B); tail with polyglactin filament (C and D); tail after removal of a titanium implant (E and F). Photographs were taken using a 4x microscope objective magnification (A, C and E) and 20x magnification (B, D and F), respectively. The scale bar corresponds to 100 µM for A, C and E; and to 200µM for B, D and F. The labeled structures shown are as follows: Adipose cells (a); tail bone with marrow (b); fibrous tissue (f), granular tissue (g); hair follicle (h); void after removal of the implant (i); muscle fibers (m) remnants from the implant interface (r); blood vessels (v).

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