In Vitro and In Vivo Biocompatibility Testing of Absorbable Metal Stents

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Summary: Permanent metallic stent implants are the most frequently applied treatment option in angioplastic interventions. Here we report *in vitro* and *in vivo* characteristics of the newly developed absorbable metal stent (AMS), consisting of magnesium (Mg) with yttrium and rare earth additives, in comparison to the clinically applied steel stent (316L). Viability and proliferation of smooth muscle cells (SMC) and endothelial cells (EC) exposed to various Mg-alloys were analyzed. AMS and 316L were implanted into coronary arteries of Göttingen minipigs (n = 17) and explanted after 28 and 56 days. Stented arterial segments were analyzed prior to explantation by angiography and *ex vivo* by micro-computer tomography (micro-CT), by histology, and by immunohistochemistry. Exposure to the alloy *in vitro* resulted in reduced viability and proliferation of smooth muscle cells. Consistently, larger luminal diameter and less neointimal formation were found in AMS both 28 and 56 days after implantation. Histological analysis revealed no significant differences in the number of macrophages and Ki-67 positive cells, yet a significantly higher number of proliferating cells around 316L struts at the earlier time point. The reduction in neointima formation and a larger vessel lumen indicates the superiority of the recently developed AMS over commercial available steel stents. Furthermore, AMS is absorbable, and thus will not cause stress to the stented vessel for the lifetime of the recipient.

Keywords: biocompatibility; biodegradable; cardiovascular; magnesium; stent

Introduction

At present, balloon dilatation followed by stent implantation is the most frequently applied treatment option in percutaneous coronary interventions. A major drawback of this technique is the development of in-stent restenosis (ISR),[1] the closure of the artery within the stented region, which occurs in about 15–25% of all cases.[2] ISR is triggered by the implantation of foreign material,[3] causing an injury which induces hyper-proliferation of vascular smooth muscle cells and inflammatory reactions.[4,5] The result is an excessive growth of the newly forming tissue layer (neointima). The long term outcome further depends on mechanical stress due to the rigidity of the implanted stent within the wall of the periodically moving artery,[6] the biocompatibility of the stent material,[7] the stent design, and the individual patient (reviewed in[8]). One possibility to overcome what is suspected to be one of the most important factors, mechanical stress,[9] is to use self-destructing stents which degrade, dissolve, or fractionate after becoming covered by the vascular wall cells.[10] As several attempts to use organic polymeric materials for this purpose were of limited success,[11–13] we tried another approach. We introduced a biodegradable metal stent

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(AMS) consisting basically of magnesium, a material which, as Mg^{2+}-ions, is abundantly present in human tissues and has important functions in the regulation of such physiological processes as cellular proliferation.[14] Mg^{2+} is well tolerated when given parenterally even at high infusion rates up to 0,5 mol/l, and features antiarrhytmic[15] and antithrombotic[16] actions. Particularly the last property is of paramount relevance in respect to the clinical performance of a stent. Data regarding performance and biocompatibility of the AMS in comparison to the stainless steel stent (316L) are presented in this article.

Methods

Cell Culture, Proliferation and Viability

Human primary smooth muscle cells (SMC) and endothelial cells (EC) derived from coronary arteries were obtained from Promocell (Heidelberg, Germany). SMC and EC were cultured in smooth muscle cell growth medium 2 (Promocell) or endothelial cell growth medium MV (Promocell), and provided supplements according to the manufactures instructions. Cells were used only up to passage 6. Extracts from tubes of different Mg alloys (3mm diameter, 4mm length, sterilization with ethylene oxide) were prepared by incubation with EC or SMC culture medium for 13 days at 37 °C, 5%CO_2 and 95% humidity. The cells were seeded into 24-well plates (2×10^4 cells/well) in duplicates for two days at 37 °C, 5% CO_2 and 95% humidity. The cell culture medium was replaced by a 1:1 dilution of extracts in EC or SMC medium, and the cells were further incubated for 4 days. Proliferation was determined by 5-Bromo-2′-deoxy-uridine (BrdU) incorporation for 90 minutes (5-Bromo-2′-deoxy-uridine Labelling and Detection Kit I, Roche), and the viability by two hours incubation with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; (MTS, CellTiter 96R AQueous One Solution Cell Proliferation Assay, Promega). Data analysis was performed according to manufactures' instructions.

Surgical Procedure, Stenting, and Tissue Processing

Degradable (AMS, Lekton Magic, n = 21) and permanent (316L, Lekton n = 10) stents (Biotronik GmbH, Berlin, Germany) were implanted in the coronary arteries of Göttingen minipigs (20–30 Kg) following a standard procedure described elsewhere.[17] The experimental protocols were performed in accordance with the local regulations and approved by the responsible animal care committee. In brief, the pretreated and sedated animals received an intravenous access line and were anaesthesized with isoflurane after intubation. The balloon-expandable AMS and the silicon carbide-coated stainless steel control (316L) both were designed for a diameter of 3 mm (10 mm length, 120 μm strut thickness, 4–5mg) after expansion. AMS were laser cut from an extruded tube of Mg-alloy. After polishing (and coating in case of Lekton), the stents were mounted by mechanical crimping on the balloon of a percutaneous transluminal coronary angioplasty catheter and γ-sterilized. Radiopaque markers were located at both ends of the balloon. After surgical placement of an introducer sheet in the right carotid artery, the stents were randomly implanted under angiographic control into the right (RCA), left anterior descendent (LAD), and the left circumflex (RCX) coronary arteries by dual balloon inflation (12at). The precise position was chosen after an initial angiography to achieve an oversize of 10–20% (expanded balloon: native vessel $\approx$ 1,1–1,2). Angiography was performed with a Phillips DCI System at implantation, after a 28 day follow up, and on explantation after eight weeks in 10 animals (5 × 316L, 10 × AMS). Seven animals (5 × 316L, 14 × AMS) were additionally sacrificed after 7 days and 4 weeks for micro-CT analysis and histological workup, respectively. Vessel reference diameters, area stenosis, and minimal luminal diameter (MLD) were measured.

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On explantation, the stented arteries were formalin-fixed by pressure-perfusion, resin embedded in methyl methacrylate (MMA) as described previously,[17] and examined by micro-CT (Fox-System, Feinfocus/Comet Garbsen, Germany). The MMA-embedded tissue blocks were further cut into 5 μm slices for histological, morphometric, and immunohistological assessment.

Histological Staining and Morphometry

Staining of the sections was achieved by adaptation of a histological standard technique (Elastica van Gieson (EvG)). The area determination of vessel wall layers and injury score (IS) was performed by applying the method of Schwarz et al.[3] using a computerized planimetry system connected to an Olympus BX41 microscope.

Immunohistological Staining

Primary antibodies were obtained from Biomeda (Mac387L1[18]) and Labvision (Ki-67[19]), horseradish peroxidase coupled secondary antibodies from Dako and Medac. Immunohistochemical staining of MMA-embedded tissues was performed according to manufactures' instructions in conjunction with heat-(Ki-67: 5 min. 0,1 M citrate buffer pH 6,0, 95 °C) or protease-(Macrophage L1: 10 min. 0,1% Protease XIV (Sigma), 37 °C) epitope retrieval. 3,3-Diaminobenzidine (DAB, Dako) was used as chromogen. The proliferation rate of media and neointima was determined by manual counting of at least 200 cells in 4–6 randomly selected fields at 50× magnification, and expressed as number of proliferating cells/number of cells ×100. The number of Mac387L1 positive macrophages was counted per arterial section.

Statistical Analysis

Statistics were performed using SPSS for Windows ® Ver. 6.0.1, (SPSS Inc. Chicago, Ill.) Results are expressed as mean ± standard deviation (SD). ANOVA with Bonferroni correction was applied to morphometric data. Angiographic data were tested by analysis of covariance (ANCOVA), using reference diameters as covariates to minimize calibration errors. Mann-Whitney nonparametric tests were used for immunohistologic data (Ki-67). Differences were considered significant if p values were less than 5%.

Results

In vitro Experiments

The proliferation and viability of EC and SMC in the presence of extracts of various

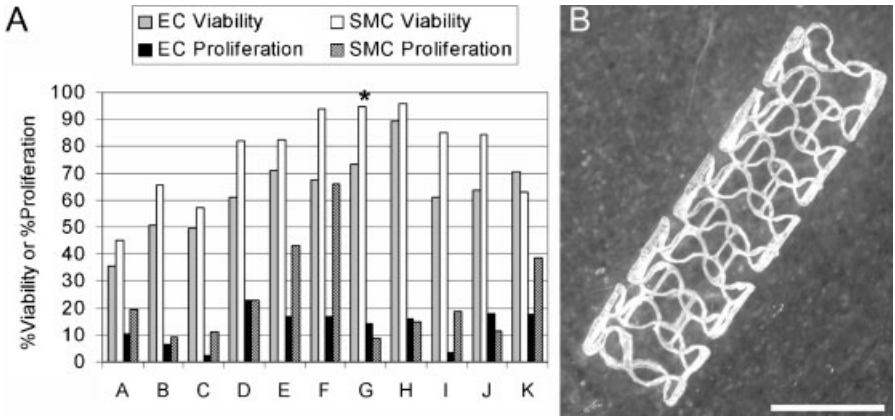

Figure 1.
A. Proliferation and viability of EC and SMC subjected to extracts of different Mg-alloys (A-K) for four days (representative experiment). Control cells = 100%. *Mg-Alloy chosen for *in vivo* experiments. B. AMS explanted after 7 days follow up (arterial tissue removed). Bar: 3mm.

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Mg-alloys were tested in an initial *in vitro* screening (Figure 1). The chosen alloys contained non-toxic or low toxic elements.[20]

Mg-alloys with additives of yttrium and rare earth elements showed a strong inhibition of SMC proliferation (approximately 80%) but a moderate reduction of SMC viability (approximately 20%), while EC were vital and showed proliferation. Additives of zinc and manganese had negative effects on viability and proliferation of EC and SMC (Figure 1A, A–C). For further in vivo studies, alloy G was chosen for the fabrication of the AMS.

In vivo Experiments

All stents were placed successfully. Angiography revealed a significantly higher MLD for AMS compared to 316L at 28 (1,51±0,46 vs 1,26±0,41 mm, $p < 0,05$) and 56 days (1,55±0,51 vs 1,09±0,25 mm, $p < 0,05$, Figure 2). For both materials, there was no further significant MLD loss from 4 to 8 weeks follow up. Angiographic stenosis was not statistically different between AMS and 316L groups (data not shown).

The neointimal areas of AMS stented arteries were significantly reduced compared to 316L stents (28d: 1,14±0,92 vs 2,35±1,41 mm^2, $p < 0,01$; 56d: 1,23±0,55 vs 2,90±0,91 mm^2; $p < 0,0005$, see Figure 3A, B and C). The IS (Figure 3D) tended to be non-significantly (N.S.) smaller for AMS at both time points (28 days: 0,37±0,72 vs 0,77±0,86; 56days: 0,12±0,24 vs 0,52±0,64). There were no significant differences between intimal areas between 4 and 8 weeks and a non- significant time trend for the IS, showing lower values at 8 weeks.

Ex vivo micro-CT analysis showed an intact stent structure at day 7, a fractionated strut structure at day 28, and an advanced degradation at day 56 (Figure 4) after implantation. All stent struts were completely covered by a dense cellular layer in the histologic sections after 7 days (data not shown). The histological sections showed an advancing strut degradation paralleling the mechanical destruction (data not shown).

Immuohistological Studies

Proliferation (Ki-67) and inflammation (monocytes/early inflammatory macrophages, L1) were evaluated by immunohistological staining of arterial sections. The rate of proliferating cells did not differ significantly in arteries with AMS compared to 361L 28 (7,25%±7,03% n = 7 for AMS vs 10,68%±5,98% n = 3 for 316L) and 56 days (5,78%±5,19% n = 4 for AMS vs 1,97%±0,67% for 316L) after implantation. A significant decrease of the proliferation rate was observed for 316L stented arteries at four to eight weeks after implantation. In the neointima, Ki-67 positive

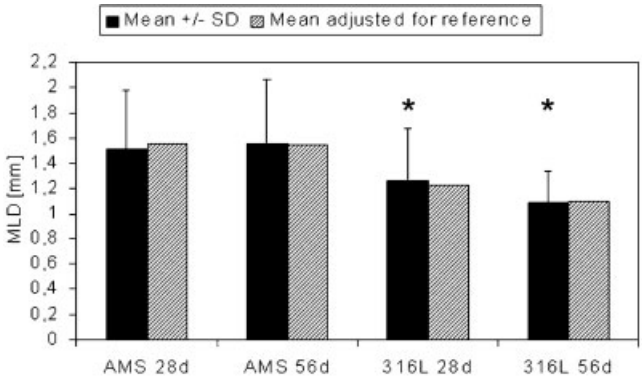

Figure 2.
MLD of AMS and 316L stented vessels 28 and 56 days (d) after implantation. * Significantly different from AMS ($p < 0,05$).

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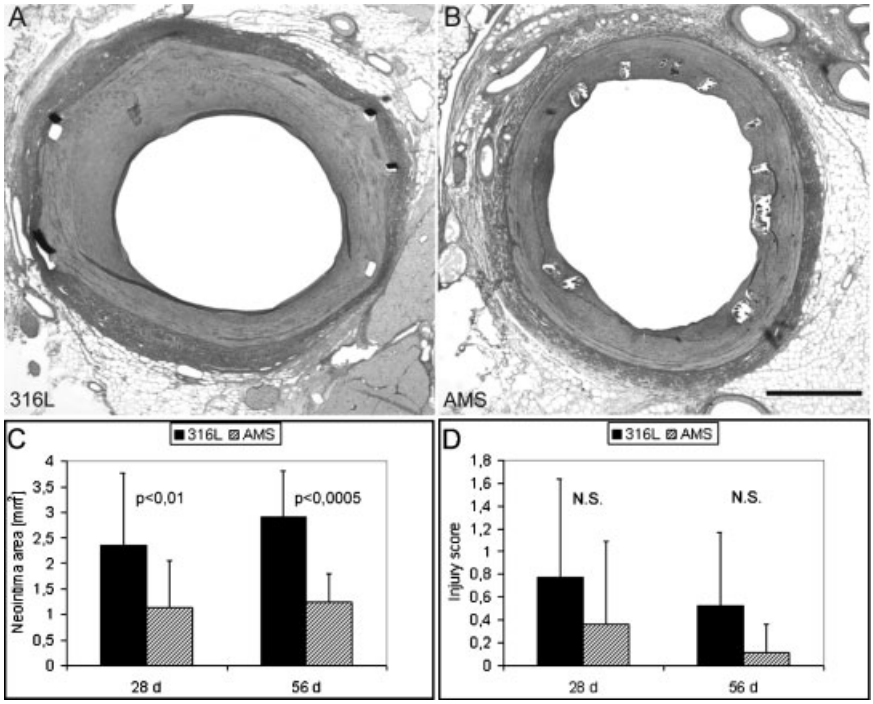

Figure 3.
316L (A) and AMS (B) stented arteries eight weeks after implantation (EvG). Bar: 1mm. Mean +- SD of neointima area (C) and injury score (D) of vessels with 316L or AMS 28 and 56 days after implantation.

cells were mainly located at the stent struts. Few Mac387L1 positive cells were detected. The number of early macrophages per artery section also did not differ at both time points (28 days: 7,7±12,4 for AMS, n = 9 vs 6,8±8,4 for 316L, n = 5 and 56 days: 8,1±9,0 for AMS, n = 7 vs 22,5±18,6 for 316L n = 4).

Discussion

Since there is an urgent need for a solution to the problem of late ISR, and even the latest developments in the area of drug delivering stents seem to have some essential drawbacks,[21] we developed and tested degradable stents fabricated from an

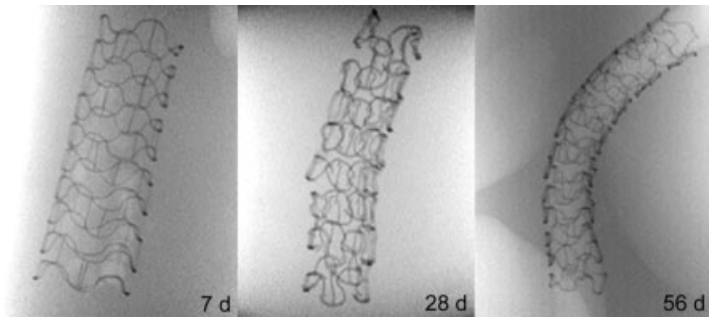

Figure 4.
Micro-CT analysis showed a proceeding AMS fractionation and degradation from seven to 56 days. Changes are indicated by a more diffuse image of the stent mesh and a reduced diameter, the latter caused by contraction of the vessel.

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Mg-alloy. Pure Mg is biocompatible, but extremely unstable in body fluids and the mass of a typical stent (5 mg) is degraded within a few seconds. Thus the use of Mg-alloys can not be avoided. Because many chemical elements in industrially available alloys are toxic to cells, we first tested the component metals for biocompatibility. It was not necessary to completely exclude an inhibiting effect on the proliferation of some cell types, as the hyperproliferation of SMCs is one of the main causes of the ISR.[1] As a consequence of our in vitro studies as well as a preliminary *in vivo*-experiment,[17] we assumed that an industrially available alloy without aluminium but containing small amounts of yttrium and rare earth elements would be most appropriate. In an initial feasibility study[17] we used a very simple production method and a rough mechanical design with a bulky structure, but we were now provided with more advanced devices by Biotronik GmbH with physical properties very similar to 316 L-stents clinically used at present. AMS have been consistently shown to induce a less intense neointimal proliferation in the *in vivo* experiments published to date,[17,22] so we tried to uncover mechanisms responsible for the reduced neointimal proliferation. We confirmed in our animal experiments the significantly reduced intimal formation in AMS, while the angiographic data were comparable. The latter fact is assumed to be caused by the physical destruction (Figure 4) and thus the mechanical incompetence of the AMS, allowing for vessel contractions (Figure 4, 56 days) and for a positive remodelling at later time points.[17,22] The non-significant difference between AMS and 316L in the proliferation activity (Ki-67) corroborates the tentative assumption that the differences in proliferation rates had vanished and the higher neointimal area in 316L stented arteries had already accumulated as early as 28 days after implantation. The significantly higher Ki-67 values at 28 days in arteries with 316L may be interpreted as an afterglow of the more intense proliferation during the first four weeks.

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