Degradation of Magnesium Alloys in Biological Solutions and Reduced Phenotypic Expression of Endothelial Cell Grown on These Alloys

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Abstract—**Generally, inflammatory response to the metal stents contributes to the formation of in-stent restenosis. However, recent development of biodegradable stents made of magnesium alloys has a potential to overcome this drawback. Nevertheless, the degradation profile of such stents and the influence on the endothelial cells remained unclear.**

In this study, the flat magnesium alloyed sheets cut by wire electrical discharge machine were immersed in distill water or culture medium. In addition, human aortic endothelial cells (HAEC) were seeded (800 cells/mm^2) onto various magnesium alloyed sheets, including Mg-Al-Zn alloys (AZ31, AZ91) and Mg-Al-Mn alloy (AM60). Cells seeded onto tissue culture treated polystyrene dish coated with gelatin were used as controls. Forty-eight hours later, the cells were examined by immunofluorescence microscopy.

The results were shown that content of Mg^{2+} within 15 weeks gradually increased to more than 40 mg/dL in the culture medium, but remained less than 10 mg/dL in the water. For all magnesium groups the cellularity at 48 hours was less than 60% of that of the controls ($p<0.01$). Comparison among the magnesium alloyed groups showed that both AZ31 and AM60 have lower values of cellularity, compared to the AZ91 (AZ31: 164 cells/mm^2; AM60: 318 cells/mm^2; AZ91: 442 cells/mm^2; AZ91 vs either AZ31 or AM60, both $p<0.05$). Immunfluorescence microscopy showed that cells grown on such magnesium metals expressed less amounts of Von Willebrand factor (VWF), connexin43 (Cx43) gap junctions, and endothelial nitric oxide synthase (eNOS).

The conclusion suggested that degradation of magnesium alloys are enhanced in culture medium, in which HAEC's had a retarded growth and protein expression profile grown on the metal. Otherwise, it also suggested that strategies to improve the biocompatibility of stents made of magnesium alloy are necessary.

I. INTRODUCTION

Although endothelial cells play a critical role during neointima formation post vascular stenting, the behavior of endothelial cells on coated stent surface was unclear.

Basically, laboratory investigation has demonstrated that endothelial cells are involved in the regulation of thrombosis and proliferation of subjacent smooth muscle cells. In addition, complete coverage of endothelial cell is associated with attenuation or even stop of the growth of neointima in the injured segment.

Although, in-stent restenosis after stenting in the coronary artery is a major drawback of percutaneous coronary intervention using stent, inclusive of drug-eluting stent (DES), owing to the dysfunction of endothelial cells are affected by the local environment. Therefore, in order to realize the biocompatibility of cells grown on magnesium alloyed materials, we examined the growth profiles of HAEC in the present study. The expression of Cx43 gap junctions, eNOS, and endothelial marker VWF were evaluated. Previous studies have shown that eNOS expressed usually in the endothelial cell of vascular tissue. Otherwise, adequate generation of nitric oxide by the eNOS is essential to the homeostasis of circulation. In addition, reduction of the eNOS and hence its product nitric oxide (NO) is well known to signify endothelial dysfunction

II. MATERIALS AND METHODS

Metal Sheets: Those metallic sheets, measuring 5 mm × 5 mm × 0.1 mm, made of various magnesium alloys were used. Afterward the surface of magnesium sheet was polished by 600 grit enamel paper and down to 0.3μm Al_2O_3 powder and cleaned by ethanol.

Cell Culture: HAEC cells of passage 4 were seeded (800 cells/mm^2) onto the metallic sheets, which were placed at the center of a 35 mm tissue culture treated polystyrene dish filled with 8 ml of culture medium (Medium 200 (GIBCO)). Forty-eight hours later, the cells were examined by immunofluorescence microscopy. For immunolabeling, cells grown on gelatin-coated (Merck, Darmstadt, Germany) glass coverslips were used as control.

Immunocytochemistry: For Cx43 and VWF double immunolabeling, cells grown on the metallic sheets were fixed with methanol at -20 °C for 5 minutes. After blocking with 0.5% BSA for 15 minutes, the cells were incubated with the anti-VWF antibody (1:50) and anti-Cx43 antibody (1:100) at 37 °C for 2 hours, followed by incubation with a CY3 conjugated donkey anti-mouse antibody (1:500; Chemicon) for 50 minutes in the dark. The cells next were incubated with bisbenzamide (1□g/ml; Sigma) for 15 minutes in the dark. Moreover, for eNOS immunolabeling, cells grown on the metallic sheets were fixed with 4% PFA

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for 5 minutes. After blocking with 0.5% BSA, the cells were incubated with the mouse anti-eNOS antibody (1:100) in the dark overnight, followed by incubation with a CY3 conjugated donkey anti-mouse antibody (1:500; Chemicon). Then, the cells were incubated with bisbenzamide. All phenotypes were mounted, examined, and recorded using an epifluorescence microscope equipped with a digital camera.

Degradation: The degradation test was carried out at room temperature using a culture medium 200 of 50 ml tube (Falcon USA). The samples, measuring 20 mm × 20 mm × 0.1 mm, made of various magnesium alloys were cleaned before testing using ethanol and immersed in the medium. Then, at each time-critical observation, 1.5 ml medium was drawn out to determine the concentration of Mg^{2+} ion within 15 weeks.

Analysis: For comparison of cellularity, cells were stained with bisbenzamide to make the nucleus visible under fluorescence microscope and were observed in x160 magnification. Images of 10 randomly selected rectangular fields. The images were then analyzed using QWIN image analysis software (Leica) to count the nucleus. For this purpose, 4 separate experiments of cell culture for all types of materials were conducted. Data, expressed as mean values (±SD), were compared statistically by t-test. A p value < 0.05 was considered to be significant.

III. Results

Determination of magnesium degradation: The content of Mg^{2+} within 15 weeks gradually increased to more than 40 mg/dL in the culture medium, but remained less than 10 mg/dL in the water (Fig. 1). The images expressed that magnesium alloy AZ31 was degraded faster than AM60 and AZ91 in medium (Fig. 2). Othetwise, these images also showed that all magesium alloys in medium were found some crystal products on the surface. On the contrary, the magesium alloys just resulted a gray film upon the surface in the water group (Fig. 3).

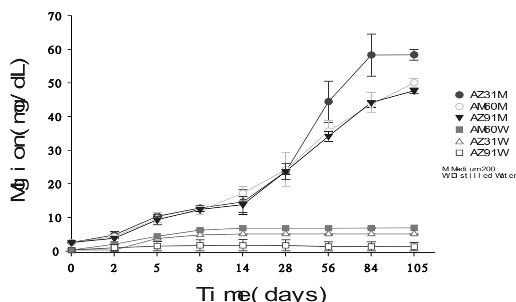

Fig. 1 Degradation rate of magnesium alloys in solution within 15 weeks.

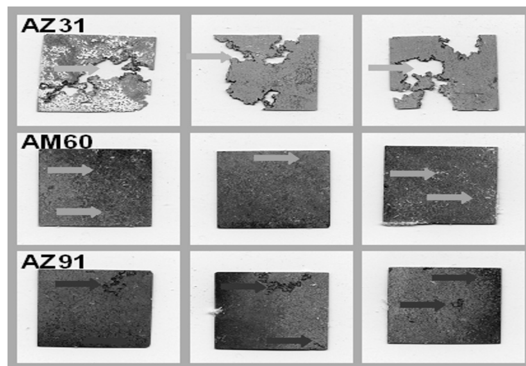

Fig. 2 The expression of magnesium alloys in culture medium 200. White small grains on the surface are crystal products.

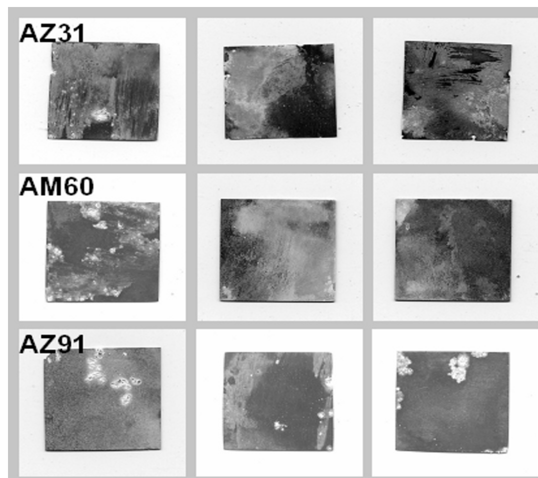

Fig. 3 The expression of magnesium alloys in the water. Gray films on the surface are oxides.

Cell Proliferation Assays: After seeding, cells were able to attach and grow on the surface, but the cell proliferation at 48 hours following the seeding varies widely, according to the magnesium alloys (Fig. 4 and Fig. 5). Regarding the magnesium alloys, for seeding of 800 cells/mm^2, all magnesium alloys have comparable or lower values of cellularity, compared to the control (all vs control, $p<0.05$). Otherwise, comparison among the magnesium alloyed groups showed that both AZ31 and AM60 have lower values of cellularity, compared to the AZ91. Generally, for the seeding of 800cells/mm^2, the cellularity on each of magnesium groups is less than 60% of control groups.

Immunofluorescence microscopy: Typically Cx43 gap junctions, seen as punctate labelngs at cells borders, were abundant in the control group, but less expressed in the remaining groups (Fig. 6). A similar pattern of expression levels was seen for VWF (Fig. 6), the labellings of which are located in the cytoplasmic compartment. Moreover,

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typically eNOS, located in the cytoplasmic compartment, was more abundant in the control group, but less expressed in the remaining groups (Fig. 7).

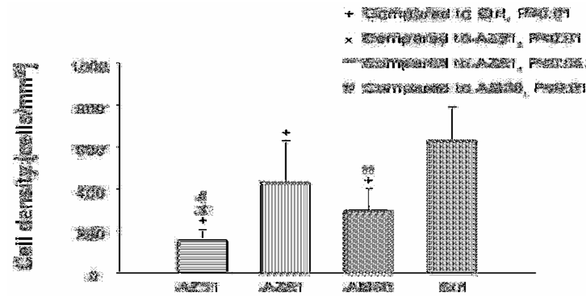

Fig. 4 Cellularity, expressed as mean ± SD, were averaged from at least 3 separate experiments and compared statistically by t-test.

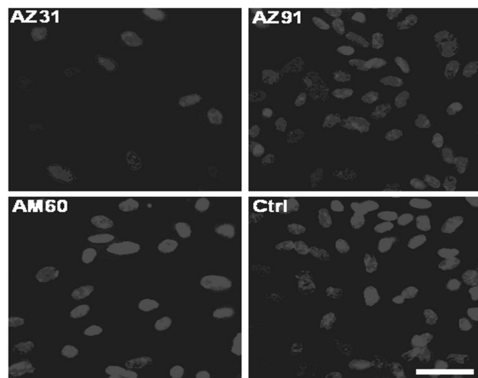

Fig. 5 Blue signal is nucleus. Bar, 100μm.

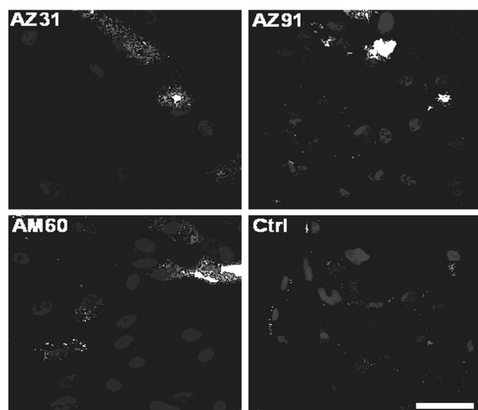

Fig. 6 Red signal is Cx43, green signal is VWF, and blue signal is nucleus. Bar, 100μm.

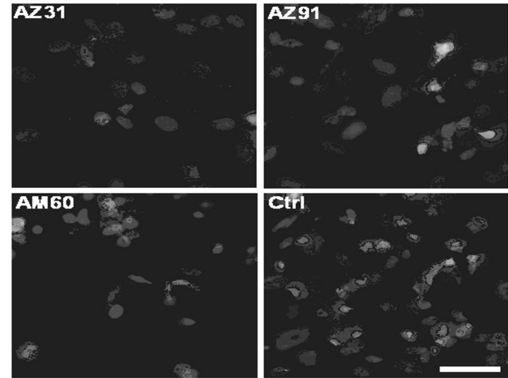

Fig. 7 Red signal is eNOS and blue signal is nucleus. Bar, 100μm.

IV. Discussion

This study demonstrates that the phenotypic features of endothelial cells, including growth profile and expression of proteins are altered when grown on the biodegradable magnesium surface. However, the finding that cells seeded on all magnesium metals, regardless of the alloyed compounds, express a small amount of Cx43, VWF, and eNOS suggest that the overlying endothelial cells have functional defects.

In addition, the corrosion rate of magnesium in such environment is very according to the magnesium percentage of alloy and it s immersed component of solution. Furthermore, when magnesium exposed to a typical atmosphere will develop a gray oxide film of magnesium hydroxide ($Mg(OH)_2$) on the surface of magnesium. However, if chloride ions are present at levels on the order of 150 mmol/L in aqueous environment, $Mg(OH)_2$ will react with Cl^- to form the magnesium chloride and hydrogen gas. Previous studies showed that pitting of magnesium was observed for Cl^- concentrations exceeding 30 mmol/L.

V. Conclusions

The degradation of magnesium alloys are enhanced in culture medium, in which HAEC s grown on the metal had a retarded growth and protein expression profile, suggesting that strategies to improve the biocompatibility of stents made of magnesium alloy are necessary. In this present study, HAEC grown on magnesium sheets vary widely in growth profile and protein expression according to the alloys. Endothelial cells grown on magnesium materials showed growth retardation and phenotypic changes, furthermore, down-regulation of Cx43, VWF, and eNOS are the common phenomena in the cells in such environments.

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These suggest the endothelial cells in the stented vessels were functionally impaired, which potentially contributes to the formation of in-stent restenosis and thrombosis post biodegradable magnesium stent implantation. Therefore, improve the biocompatibility of stents made of magnesium alloy are necessary.

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