An In Vitro Study on Early Inflammatory Response to Four Magnesium-Zinc-Strontium Alloys

Aaron F. Cipriano[1,2], Amy Sallee[1], Renguo Guan[4], and Huinan Liu[1,2,3]
[1]Dept. of Bioengineering, [2]Materials Science & Engineering Program, [3]Stem Cell Center, University of California, Riverside CA 92521 USA. [4]School of Materials and Metallurgy, Northeastern University, Shenyang 110004, CHINA

Statement of Purpose: Magnesium-Zinc-Strontium (Mg-Zn-Sr) alloys are currently being investigated for a variety of biomedical applications due to the natural presence of all of the alloying elements in the human body and to the desirable mechanical/corrosion properties of the alloys [Cipriano AF. J. Mater Sci Mater Med **(2013)** 24: 989-1003, 2013; Liu L. Med Sci Monit **(2014)** 20: 1056-1066]. It is idealized that, in situ, the slowly-degrading Mg-based implants will be first accompanied by the initiation, resolution, and organization of the acute and chronic inflammatory host response to the degradation products [Witte F. JBMR A **(2007)** 81: 748-756]. The objective of this study was to investigate the cytocompatibility and degradation of four Mg-4Zn-xSr alloys (x = 0.15, 0.5, 1.0, 1.5 wt. %; designated as ZSr41A, B, C, and D respectively) and their effects on adhesion and expression of vascular cell adhesion molecule 1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs) after 4 hrs of incubation. Endothelial cell activation (type II) is characterized by the upregulation of pro-inflammatory proteins such as VCAM-1 [Ley K. Nat Rev Immunol **(2007)** 7: 678-689]; therefore, the expression of VCAM-1 was used as a marker for inflammation in this study.

Methods: HUVECs (Lonza Walkersville Inc.) were cultured according to the manufacturer's directions and co-seeded (P5) directly with the samples at $6x10^3$ cells cm^{-2} and incubated in EGM-2 supplemented media (Lonza Walkersville Inc.) under standard cell culture conditions for 4 hrs. Commercially available pure Mg (99.95%, as rolled; Alfa Aesar) was used as a reference material and HUVECs cultured in EGM-2 media supplemented with 10 ng/mL of tumor necrosis factor α (TNFα; Life Technologies) was used as a positive control for VCAM-1 expression (POS). For immunofluorescence, cells adhered to the culture plates were fixed with 4% formaldehyde for 20 min, incubated (blocked) with 5% goat serum in PBS for 1 hr, incubated with 10 µg/mL VCAM-1 (Santa Cruz Biotechnology Inc.) mouse anti-human primary IgG overnight at 4 °C, and incubated with 10 µg/mL goat anti-mouse secondary IgG-FITC (Santa Cruz Biotechnology Inc.) at room temperature for 1 hr. The nuclei were subsequently stained with 4',6-diamidino-2-phenylindole dilactate (DAPI; Life Technologies) nucleic acid stain for 5 min. HUVECs-only (no stimulation) were fixed and stained as described above and used as a negative control for VCAM-1 expression (NEG). Five FITC images (640x512 pixel digital resolution, 16-bit depth, and 4 s exposure) per well per group were captured using a fluorescent microscope (Nikon Eclipse Ti, Nikon Instruments). A mean fluorescence intensity signal per pixel was calculated using the method described herein (Patapova TA. Mol Biol Cell **(2011)** 22: 1191-1206). Additionally, DAPI-stained nuclei were observed and counted per unit area to determine cell adhesion density.

Results: Adherent and viable cells were observed on the culture plates of all samples; the morphology of these adhered cells appeared normal and healthy (**Figure 1a**). VCAM-1 expression was significantly lower for ZSr41A when compared with ZSr41D and POS; likewise, VCAM-1 expression was significantly lower for NEG when compared with ZSr41D and POS (**Figure 1b**). Finally, no significant differences were found for HUVEC adhesion density after 4 hrs of incubation (**Figure 1c**).

Conclusions: ZSr41 alloys studied showed adequate cytocompatibility with HUVECs after 4 hrs of incubation since cell adhesion density was not significantly different for any of the groups. VCAM-1 expression of viable and adherent cells had a dependence on alloy type. Future work will focus on: (i) elucidating whether VCAM-1 up-regulation is modulated by alloy degradation rate or Sr content, and (ii) evaluating HUVEC adhesion, morphology, and VCAM-1 expression at 24 hr.

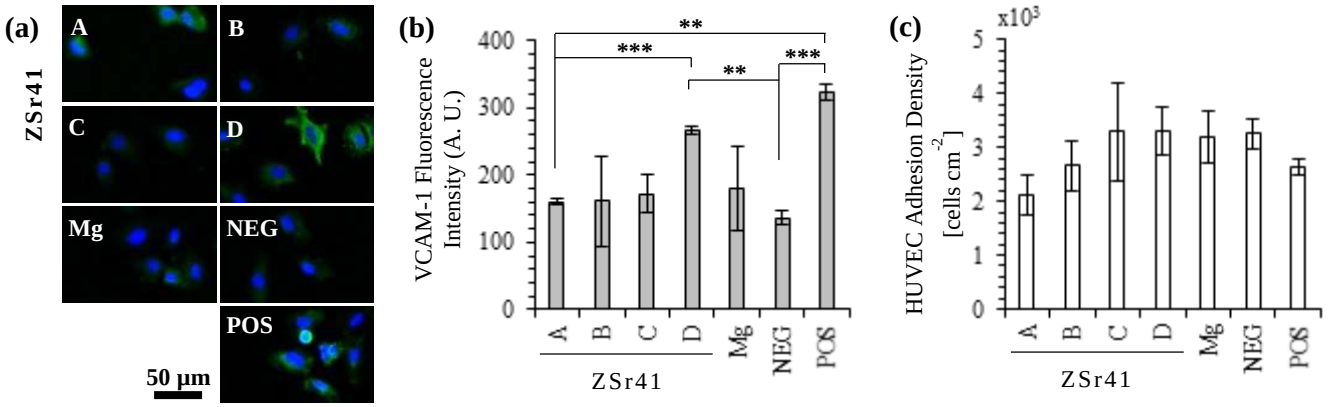

Figure 1: HUVEC responses at 4 hr direct cultures with ZSr41A-D alloys, pure Mg reference, cells only negative control (NEG), and cells stimulated with TNFα (positive control, POS): **(a)** Fluorescence images of adhered HUVECs. Blue color indicates DAPI stained nuclei and green color indicates FITC-labeled VCAM-1. Scale bar = 50 µm for all images. **(b)** Quantification of VCAM-1 mean fluorescence intensity signal per pixel. **(c)** HUVEC adhesion density. All results only for cells adhered on the culture plate surrounding the samples. **Statistical analysis:** All experiments were run in triplicate. Parametric data sets were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey HSD post hoc test. Data sets with normal distribution but heterogeneous variance were analyzed using one-way ANOVA (homogeneous variance not assumed) followed by the Games-Howell post hoc test. Statistical significance was considered at $p < 0.05$. Values are mean ± standard error of the means; n = 3; $^{**}p < 0.01$, $^{***}p < 0.001$.