Acta Biomaterialia 48 (2017) 499–520

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Cytocompatibility and early inflammatory response of human endothelial cells in direct culture with Mg-Zn-Sr alloys

Aaron F. Cipriano [a,b], Amy Sallee [a], Myla Tayoba [a], Mayra C. Cortez Alcaraz [a], Alan Lin [a], Ren-Guo Guan [c], Zhan-Yong Zhao [c], Huinan Liu [a,b,d,e,*]

[a] Department of Bioengineering, University of California, Riverside, CA 92521, USA
[b] Materials Science & Engineering Program, University of California, Riverside, CA 92521, USA
[c] School of Materials and Metallurgy, Northeastern University, Shenyang 110004, China
[d] Stem Cell Center, University of California, Riverside, CA 92521, USA
[e] Cell, Molecular and Developmental Biology Program, University of California, Riverside, CA 92521, USA

ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form 4 October 2016
Accepted 12 October 2016
Available online 13 October 2016

Keywords:
Biodegradable Magnesium Zinc Strontium alloy
Mg-Zn-Sr alloy
Human umbilical vein endothelial cells (HUVEC)
Induction of vascular cell adhesion molecule-1 (VCAM-1)
Early *inflammatory response*
Biomedical implants

ABSTRACT

Crystalline Mg-Zinc (Zn)-Strontium (Sr) ternary alloys consist of elements naturally present in the human body and provide attractive mechanical and biodegradable properties for a variety of biomedical applications. The first objective of this study was to investigate the degradation and cytocompatibility of four Mg-4Zn-xSr alloys (x = 0.15, 0.5, 1.0, 1.5 wt%; designated as ZSr41A, B, C, and D respectively) in the direct culture with human umbilical vein endothelial cells (HUVEC) *in vitro*. The second objective was to investigate, for the first time, the early-stage inflammatory response in cultured HUVECs as indicated by the induction of vascular cellular adhesion molecule-1 (VCAM-1). The results showed that the 24-h *in vitro* degradation of the ZSr41 alloys containing a β-phase with a Zn/Sr at% ratio ~1.5 was significantly faster than the ZSr41 alloys with Zn/Sr at% ~1. Additionally, the adhesion density of HUVECs in the direct culture but not in direct contact with the ZSr41 alloys for up to 24 h was not adversely affected by the degradation of the alloys. Importantly, neither culture media supplemented with up to 27.6 mM Mg^{2+} ions nor media intentionally adjusted up to alkaline pH 9 induced any detectable adverse effects on HUVEC responses. In contrast, the significantly higher, yet non-cytotoxic, Zn^{2+} ion concentration from the degradation of ZSr41D alloy was likely the cause for the initially higher VCAM-1 expression on cultured HUVECs. Lastly, analysis of the HUVEC-ZSr41 interface showed near-complete absence of cell adhesion directly on the sample surface, most likely caused by either a high local alkalinity, change in surface topography, and/or surface composition. The *direct* culture method used in this study was proposed as a valuable tool for studying the design aspects of Zn-containing Mg-based biomaterials *in vitro*, in order to engineer solutions to address current shortcomings of Mg alloys for vascular device applications.

Statement of Significance

Magnesium (Mg) alloys specifically designed for biodegradable implant applications have been the focus of biomedical research since the early 2000s. Physicochemical properties of Mg alloys make these metallic biomaterials excellent candidates for temporary biodegradable implants in orthopedic and cardiovascular applications. As Mg alloys continue to be investigated for biomedical applications, it is necessary to understand whether Mg-based materials or the alloying elements have the intrinsic ability to direct an immune response to improve implant integration while avoiding cell-biomaterial interactions leading to chronic inflammation and/or foreign body reactions. The present study utilized the direct culture method to investigate for the first time the *in vitro* transient inflammatory activation of endothelial cells induced by the degradation products of Zn-containing Mg alloys.

* Corresponding author at: Department of Bioengineering, University of California at Riverside, 900 University Avenue, Riverside, CA 92521, USA.
E-mail address: huinan.liu@ucr.edu (H. Liu).

http://dx.doi.org/10.1016/j.actbio.2016.10.020
1742-7061/

1. Introduction

Magnesium (Mg) alloys specifically designed for biodegradable implant applications have been the focus of biomedical research since the early 2000s [1–3]. Physicochemical properties of Mg alloys make these metallic biomaterials excellent candidates for temporary biodegradable implants in orthopedic and cardiovascular applications [1–4]. Most notable is the fact that the human body contains a large amount of Mg ions and can effectively metabolize the degradation products of Mg [1–3]. Therefore, temporary biodegradable metallic implants are idealized to be superior alternatives to permanent implants in that they would eliminate the need for implant removal surgeries following healing of the damaged tissue. By doing so, Mg-based biodegradable implants could reduce the burden on the healthcare system by mitigating risks and costs [5,6]. Furthermore, as Mg alloys continue to be investigated for biomedical applications, it is necessary to understand whether Mg-based materials or the alloying elements have the intrinsic ability to direct an immune response to improve implant integration while avoiding cell-biomaterial interactions leading to chronic inflammation and/or foreign body reactions [7,8]. In contrast, previous studies have shown that conventional permanent metallic implants, and associated wear debris, can trigger chronic inflammatory responses, result in tissue loss, and are prone to infection [1,6,9].

In general, the initial (acute) inflammatory response (i.e. innate immunity) to biomaterials is activated by the reaction of vascularized connective tissue to injury caused either by trauma or implantation [10,11]. The physiological response of inflammation consists of a complex series of meticulously controlled responses which cannot possibly be summarized in a few sentences; however, the informed reader is referred to excellent reviews on the inflammatory response to biomaterials [8,11]. Endothelial cells (EC) play an important role in the regulation of immune and inflammatory local responses by expressing, among other things, cell adhesion molecules (CAM) [12,13]. CAM expression in activated ECs (type II activation) can be induced by pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNFα) [12,13], released by inflammatory cells activated on contact with adsorbed proteins on the implanted biomaterial [8,11,14]. In turn, these adhesion molecules help recruit leukocytes from circulating blood and facilitate transendothelial migration to the site of injury to initiate the acute inflammatory response [8,11–14]. Additionally, previous *in vitro* studies showed that CAM expression in ECs was activated by elevated concentrations of metallic ions typically found in permanent metallic implants [7,15–23]. Vascular cell adhesion molecule-1 (VCAM-1) is an immunoglobulin superfamily-specific receptor that provides high-affinity interactions between ECs and integrins on the leukocyte surface and facilitates transendothelial migration [10,13,14]. Moreover, VCAM-1 binds with monocytes, but not neutrophils, and it is the first CAM expressed in chronic inflammation such as atherosclerosis (before atherosclerotic plaque development) [13,14,24] and restenosis following coronary stent implantation [25]. Thus, VCAM-1 can be used as an indicator of *in vitro* EC activation during the early stages of inflammation. Furthermore, previous studies supported the applicability of human umbilical vein endothelial cells (HUVEC) to model and investigate components of the inflammatory response, such as CAM expression [7,17].

Previously, we reported the development of Mg-Zinc-Strontium (Mg-Zn-Sr) ternary alloys and the evaluation of their biological performance for biomedical applications [26–28]. Furthermore, we reported the *in vitro* direct culture method to mimic *in vivo* physiological conditions and evaluate cell responses at the cell-biomaterial interface (*direct contact*) and on the culture plate (*indirect contact*; exposure to solubilized degradation products) surrounding the Mg-based biomaterial [29]. The direct culture method was introduced to provide a more comprehensive *in vitro* method, as compared with ISO 10993-based methods, for the initial rapid screening of cytocompatibility and degradation of Mg-based biomaterials [29]. The direct culture method was introduced as part of a field-wide effort to improve and standardize the *in vitro* testing of Mg-based biomaterials [29–32]. Thus, the first objective of this study was to investigate the degradation and cytocompatibility of four Mg-4Zn-xSr alloys (x = 0.15, 0.5, 1.0, 1.5 wt%; designated as ZSr41A, B, C, and D respectively) in the direct culture with HUVECs *in vitro*. The second objective was to investigate the induction of an inflammatory response in HUVECs as indicated by the expression of VCAM-1 activated by the degradation products of the ZSr41 alloys. While several recent *in vivo* studies reported adequate immunological response during the foreign body reaction or fibrosis stages following implantation of Mg-based materials [33–37], sparse literature is found on the early-stage inflammatory response. Specifically, to the authors' knowledge, early-stage inflammatory induction by the degradation of Mg-based materials has only been investigated *in vitro* with primary murine and human macrophages [38] and with dendritic cells [39]. In both cases, the Mg-based materials and the respective degradation products were not found to have detrimental immunomodulatory effects. This study reported for the first time on the *in vitro* transient inflammatory activation of ECs induced by the degradation products of Zn-containing Mg alloys.

2. Materials and methods

2.1. Preparation of ZSr41 alloys, Mg control, and reference materials

The ZSr41 alloys in this study had a nominal composition of 4 wt% Zn with 0.15, 0.5, 1.0, or 1.5 wt% Sr; these alloys were designated as ZSr41A, ZSr41B, ZSr41C, and ZSr41D accordingly with increasing Sr content. Details pertaining to the metallurgical process and heat treatment used for alloy preparation are described elsewhere [26,27]. The heat-treated 1.0 mm thick sheets of ZSr41 alloys were cut into 5 × 5 mm squares. Likewise, commercially pure Mg sheets (99.9%, As-rolled, 1.0 mm thick, Cat# 40604; Alfa Aesar, Ward Hill, MA, USA) were cut into 5 × 5 mm squares and used as a control in this study. Commercially available AZ31 (1.0 mm thick, Cat# 44009; Alfa Aesar) and Nitinol (NiTi; 0.25 mm thick, Cat# 44953; Alfa Aesar) sheets were cut into 5 × 5 mm squares and used as metallic reference materials in this study. AZ31 was included in this study since it has been used previously as a reference material for the investigation of Mg-based materials [40–42]; likewise, NiTi was included due to the widespread use for cardiovascular stents [43]. Additionally, 90:10 polylactic-co-glycolic acid (PLGA) was included in this study as a non-metallic reference material due to the use of PLGA-based coatings to control the degradation of Mg-based materials for cardiovascular stents [43,44]. The PLGA samples were prepared by spin coating onto the non-tissue culture treated glass (Cat# 12-544-1; Fisher Scientific, Hampton, NH, USA), which was cut into 5 × 5 mm squares followed by rough polishing with 240 grit SiC paper to improve the glass-PLGA adhesion. The 90:10 PLGA (Cat# AP49; Polyscitech, West Lafayette, IN, USA) was dissolved in chloroform at 10 wt/vol% and sonicated for 1 h at 40 °C. Subsequently, 50 μL of the dissolved PLGA was spin coated (SC100; Smart Coater Co., Saint Louis, MO, USA) onto the rough-polished glass substrates at 1300 RPM for 60 s. The PLGA coatings had a thickness of 64.0 ± 12.6 μm.

2.2. Microstructure characterization

The microstructure and surface elemental composition/distribution of the ZSr41 alloys, pure Mg control, and AZ31 reference