In vitro evaluations of innate and acquired immune responses to electrospun polydioxanone–elastin blends

Matthew J. Smith [a], Kimber L. White, Jr. [b], Donna C. Smith [b,c], Gary L. Bowlin [a,*]

[a] *Department of Biomedical Engineering, 401 W. Main Street, P.O. Box 843067, Virginia Commonwealth University, Richmond, VA 23284-3067, USA*
[b] *Department of Pharmacology and Toxicology, 527 N. 12th Street, Strauss Research Laboratories, P.O. Box 980613, Virginia Commonwealth University, Richmond, VA 23298, USA*
[c] *Altria Client Services, Richmond, VA 23231, USA*

ARTICLE INFO

ABSTRACT

Immune response testing of biomaterials is an essential component of biocompatibility assessment, particularly when the materials of interest are used to design bioresorbable scaffolds with the potential to promote *in situ* regeneration. Current trends in immune response testing of biomaterials typically examine few elements of the immune system, and they often undertake a mechanistic approach without first determining if material exposure results in physiologically relevant modulation of both innate and acquired immunity. Here, we present a comprehensive *in vitro* evaluation of biomaterial-induced modulation of acquired (i.e. cell-mediated and humoral) and innate immune responses following exposure to electrospun blends of polydioxanone (PDO) and elastin (ELAS). Results indicated that *in vitro* exposure of murine spleen cells to PDO–ELAS blends produced statistically significant immunosuppression in multiple cell-mediated and humoral endpoints. Results suggested that ELAS is the primary cause of cell-mediated immunosuppression. In contrast, PDO and ELAS were equally suppressive of humoral immune responses, while blends of the two were more immunosuppressive than either pure polymer alone. Evaluations of innate immune responses demonstrated few significant effects, with statistically significant immunosuppression observed in natural killer cell activity but not in macrophage functional assays. This work presents an approach for assessing potential modulation of immune responses resulting from exposure to biomaterials, and such evaluations are essential to obtaining comprehensive assessments of biocompatibility.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Immune response testing of biomaterials has been indicated as essential to a favorable determination in support of the contention that a given material is "biocompatible" [1]. Much published research evaluates biocompatibility in terms of cell morphology following cell seeding and culture on materials, often presenting favorable results that indicate materials are biocompatible [2–4]. Others have published regarding the critical role of the macrophage in the host response to biomaterials and the potential for modulation of macrophage cytokine production following biomaterial exposure [5–8]. The resulting body of published work, often only examining modulation of a small number of cytokines or growth factors, has little contextual foundation. While such information is certainly important, this focused approach is too narrow without first characterizing the overall effects of the biomaterial of interest on the immune responses of the host. In addition, current biocompatibility and immune response testing remains focused on inflammation, resulting in little information as to the effects of biomaterials on both acquired (cell-mediated, i.e. T-cell; and humoral, i.e. B-cell) immunity and other innate parameters, including natural killer (NK) cell activity and macrophage functions such as phagocytosis and respiratory burst.

We have recently published on the need for more comprehensive testing of immune responses to biomaterials [9]. Therein, we proposed a methodology for evaluating immune responses that has been adapted from the approach used in immunotoxicological evaluations of pharmacological compounds. This approach allows for an in-depth evaluation that is more extensive than current approaches for determining the potentially adverse effects of biomaterials on the immune system. In particular, responses following exposure to blends of synthetic and natural bioresorbable materials, such as polydioxanone (PDO) and elastin, are of interest in our laboratory.

* Corresponding author. Tel.: +1 804 828 2592; fax: +1 804 828 4454.
E-mail address: glbowlin@vcu.edu (G.L. Bowlin).

Polydioxanone (PDO) is a colorless bioresorbable synthetic poly(ether ester) which undergoes hydrolytic degradation *in vivo* and *in vitro*. In many applications, the use of PDO over other more traditional resorbable polymers, such as poly(glycolic acid), and poly(lactic-*co*-glycolic acid), is desirable because PDO is highly flexible, has a moderate degradation rate, and is believed to produce a lower inflammatory response [10]. PDO suture (PDS™-II) has been shown to produce no acute or chronic toxicological effects in any tissues or organs following a six-month *in vivo* implantation [11]. However, PDS™-II induced fibrosis after 14 days in a rat subcutaneous implantation model [12]. Uff et al. demonstrated that, while soluble suture fragments of PDO had fewer effects on the macrophage as compared to silk, nylon, and polyglactin, each of these materials were indicated to release "immunotoxic factors" [13], although no specific factors were identified. These studies indicate the importance of examining the immune responses to PDO during its degradation phase, rather than assuming all effects are transient. The promise of PDO as a less thrombogenic, non-toxic, and minimally inflammatory biomaterial with properties particularly desirable in vascular tissue engineering has led to its use in vascular prosthetic designs (woven [14,15] and electrospun [16,17]).

Elastin is one of the three essential components of native biological elastic tissue and is rich in the amino acids valine (V), glycine (G), alanine (A) and proline (P). The primary function of elastin is to provide elasticity to tissues, thus it is found primarily in arteries, lung tissue, skin, ligaments, and tendons [18]. Though highly insoluble, elastin can be processed into soluble forms via chemical hydrolysis. Hydrolysis by oxalic acid produces α-elastin, and hydrolysis by potassium hydroxide produces κ-elastin [18]. An excess of κ-elastin has been shown to induce lymphocyte apoptosis [19]. Elastin-derived peptides (EDPs), which result from the degradation of elastin during such disease states as atherosclerosis, have been shown to impact the immune system. Much published research has focused on a six amino acid repeating sequence specific to elastin, VGVAPG. This peptide sequence is immunogenic [20] and chemotactic for monocytes and fibroblasts [21,22]. Debret et al. have reported that VGVAPG peptide fragments can cause modulation of a variety of cytokines, including IFN-γ, IL-10, and IL-1 [23,24]. Specifically, they indicated that EDPs are able to skew CD4 + T-cells toward a Th1 profile [23] by increasing expression of IL-2 and IFN-γ (Th1 cytokines) and decreasing expression of IL-5 and IL-10 (Th2 cytokines) by cells pre-disposed toward the Th2 lineage, effectively reversing Th2 orientation.

In recent publications, electrospun blends of PDO and α-elastin have demonstrated significant potential for use in vascular replacement applications [16,17]. For *in situ* small-diameter vascular tissue engineering applications, grafts made with biodegradable polymers that are non-thrombogenic are desirable over other synthetics, such as e-PTFE, because over time, the body can degrade a bioresorbable graft and replace it with a newly regenerated vascular structure virtually indistinguishable from native vessels [25,26]. A graft that degrades and is replaced with newly formed tissue over time may avoid the formation of a permanent, impenetrable acellular fibrotic capsule. As such, bioresorbable tissue-engineered scaffolds have significant potential applications, however, the potential effects of these biomaterials on the immune responses of the host have not been evaluated.

The study presented here offers an *in vitro* evaluation of the immunomodulatory effects of electrospun blends of PDO and α-elastin (ELAS) on innate and acquired immune responses. Effects on components of the innate immune system were examined by evaluating NK cell activity; macrophage phagocytosis and production of reactive nitrogen and oxygen species. Modulation of cell-mediated responses were assessed by evaluating T-cell proliferation (induced by either anti-CD3 antibody or concanavalin A), the one-way mixed leukocyte response to allogenic stimulator cells, and the ability of cytotoxic T-cells to recognize, proliferate, and produce effector cells against mastocytoma tumor cells, while humoral immune effects were evaluated using B-cell proliferation assays (with proliferation induced by either lipopolysaccharide or F(ab')₂ antibody fragments) and the Mishell–Dutton *in vitro* antibody-forming cell assay.

2. Materials and methods

2.1. PDO–ELAS blend preparation and scaffold fabrication

Prior to electrospinning onto a grounded rectangular mandrel (7.7 cm × 2 cm × 0.5 cm), PDO (Ethicon, Inc., Somerville, NJ, USA) and soluble bovine ELAS (Elastin Products Co. Inc., Owensville, MO, USA) were each dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP; TCI America, Portland, OR, USA) at concentrations of 100 mg/ml and 250 mg/ml, respectively, and then blended at ratios of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 by volume. Three millilitres of each blend were drawn into a Becton–Dickinson syringe fitted with an 18-gauge blunt-tip needle and dispensed using a KD Scientific syringe pump at a rate between 2 and 8 mL/h. During electrospinning, a constant voltage between 22 and 25 kV was applied to the needle via the positive lead of a Spellman CZE1000R high voltage power supply (Spellman High Voltage Electronics Corp., Hauppauge, NY, USA). All other electrospinning parameters were kept constant, including the air gap distance (12.7 cm), mandrel translation (±3.75 cm), and the rotational and translational speeds of the mandrel (500 RPM and 2 cm/s, respectively).

2.2. Cross-linking and preparation of samples for culture

All scaffolds were treated for 18 h using a 50-fold molar excess (210 mM) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce Biotechnologies, Rockford, IL, USA) in ethanol for 18 h, followed by a 2-h rinse in 0.1 M sodium phosphate as previously described [17,27,28]. While EDC treatment of pure PDO scaffolds is unnecessary (due to the absence of natural polymers), these scaffolds were also treated in the same manner as all PDO–ELAS blends in order to allow for scientifically valid statistical comparisons between blend groups. Following cross-linking treatment, scaffolds were disinfected in ethanol for 10 min, followed by three successive 10-min rinses in sterile PBS. Finally, materials were cut into discs or squares as required for each individual assay described below.

2.3. Animal species selection and husbandry

All animal research was conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. Female B6C3F1 mice (Taconic Farms, Germantown, NY, USA) between 12 and 18 weeks of age were used in these studies. The B6C3F1 strain was selected because it is the animal of choice for immunotoxicological evaluations conducted by the National Toxicology Program. In addition, due to being an inbred strain (F1), genetic differences are minimal between animals in this strain, thus cells from multiple animals can be pooled. Mice were housed 4 animals per cage in plastic shoe box cages with hardwood bedding and were maintained on Harlan Teklad Laboratory Diet as required by the NIH. Animals were housed in an isolated vivarium located on the campus of Virginia Commonwealth University, where conditions were maintained at 21–24 °C and 40–70% relative humidity with 12 h light-dark cycles.

2.4. Spleen cell suspension preparation

As one of the primary lymphoid tissues, the spleen filters peripheral blood and consists primarily of B-cells and T-cells (lymphocytes), as well as macrophages and natural killer cells. In order to prepare a single-cell suspension of spleen cells (splenocytes), mice were euthanized by CO₂ inhalation followed by cervical dislocation, after which spleens were collected aseptically and gently mashed between frosted ends of two sterile microscope slides. Unless otherwise indicated, splenocytes were resuspended in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum (FBS), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5% sodium bicarbonate, 50 μg/ml gentamicin, and 10 μM 2-mercaptoethanol (2-ME) (complete media) prior to adjusting cell concentrations as needed.

2.5. Natural killer cell activity

The activity of NK cells exposed to blends of PDO–ELAS was measured using a modified version [29] of the assay of Reynolds and Herberman [30]. Splenocytes were adjusted to four concentrations and added in a volume of 0.1 ml to wells of a 96-well plate with or without 3-mm diameter circular punches of material. Target cells, the T cell lymphoma YAC-1, were labeled with ⁵¹Cr and then added to all wells in a volume of 0.1 ml to obtain effector:target ratios of 200:1, 100:1, 50:1, and 25:1.