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Elucidation of adhesion-dependent spontaneous apoptosis in macrophages using phase separated PEG/polyurethane films

Angela L. Zachman^{a,b}, Jonathan M. Page^c, Gayathri Prabhakar^a, Scott A. Guelcher^{a,b,c}, and Hak-Joon Sung^{a,b,d,e,*}

^aDepartment of Biomedical Engineering, Vanderbilt University, Nashville, TN 37235, USA

^bCenter for Matrix Biology, Vanderbilt University Medical Center, Nashville, TN 37235, USA

^cDepartment of Chemical and Biomolecular Engineering, Vanderbilt University, Nashville, TN 37235, USA

^dCenter for Vascular Biology, Vanderbilt University, Nashville, TN 37235, USA

^eDivision of Cardiovascular Medicine, Vanderbilt University, Nashville, TN 37235, USA

Abstract

Circulating monocytes undergo spontaneous apoptosis when there is no activation stimulus, which is critical to population control for proper host response to implants. As activation and apoptosis of monocytes/macrophages are regulated by cell–cell and cell–matrix interactions, their regulatory mechanism was investigated in this study using polyethylene glycol (PEG)-containing polyurethane films in which PEG-rich and polyester-rich domains were phase separated. Human blood monocyte-derived macrophages (HBMs) preferentially adhered to PEG domains (cell–matrix interaction) due to the low molecular weight (600 g mol⁻¹), resulting in increased HBM density (cell–cell interaction). As both cell–cell and cell–matrix interactions were promoted, HBM apoptosis increased, while their activation as measured by phagocytosis, intracellular reactive oxygen species (ROS) level and matrix metalloproteinase-9 production decreased compared to PEG-free films. When cell seeding density and cell-adhesive gelatin coating on silicone films were controlled, a cooperative role of cell–matrix (adhesion) and cell–cell (density) interactions in inducing HBM apoptosis was observed. Expression of the macrophage adhesion molecule CD11b caused apoptosis in this context, which was mediated by tissue necrosis factor- α signaling but down-regulated by the ROS inhibitor diphenylene iodonium and the anti-inflammatory peptide Ac-SDKP, suggesting a new concept for the design of biomaterials that allows for cell adhesion without excessive inflammatory activation.

Keywords

Macrophage; Inflammation; Polyurethane; Cell adhesion; Apoptosis

1. Introduction

Polymeric biomaterials can be endowed with biologically instructive properties for control of inflammatory cell activation by modulating cell–matrix and cell–cell interactions [1,2]. When non-adherent monocytes arrive at the surface of a biomaterial, they undergo activation

processes and differentiate to adherent macrophages (“cell–matrix” interaction). Activated macrophages form multinuclear giant cells (MNGCs) through fusion with neighboring cells (“cell–cell” interaction), which delays programmed cell death (e.g. apoptosis), thereby providing these highly phagocytic cells with enough time to digest and degrade the biomaterial [1].

It has been reported that free-floating monocytes undergo spontaneous apoptosis when there is no proper stimulus for activation [3]. Adhesion of macrophages to highly hydrophilic or anionic biomaterials can induce apoptosis without exacerbating inflammation, suggesting a potential means to prevent excessive foreign body response to implanted materials [2]. However, the mechanism driving spontaneous apoptosis of macrophages in interaction with biomaterials remains unclear. Additionally, such highly hydrophilic or anionic materials do not promote attachment of other desired cell types, as needed for successful tissue engineering [4,5].

In this study, we employed a novel biomaterial design to study a potential role of cell–matrix and cell–cell interactions in regulating macrophage apoptosis. Polyurethanes (PURs) are commonly utilized biomaterials with a wide range of applications. They are formed by the condensation reaction of a polyisocyanate (hard segment) and a polyalcohol (polyester, soft segment) [6,7]. As a library of PURs can provide diverse mechanical and chemical properties, these polymers are used in various tissue regeneration applications, including skin [7], bone [8], cardiovascular [9] and nerve [10]. In this study, copolymers of a lysine tri-isocyanate (LTI) (hard segment) and a mixed soft segment composed of low-molecular-weight (MW) polyethylene glycol (PEG; MW= 600 Da) and a trifunctional polyester triol (polyol; MW= 900 Da) were used. The two components of the soft segment were chosen based on their biocompatibility and well-documented immiscibility [11]. Polyester-g-PEG block copolymers are widely utilized in micelleforming applications due to their differences in hydrophilicity [11,12]. Furthermore, similarly immiscible polymers incorporated in PUR soft segments have been shown to spontaneously phase separate on the macroscopic scale [13]. It was hypothesized that, even when constrained in an interpenetrating PUR network, the polymers would phase separate into PEG-rich domains and polyester-rich domains. PEG was added into the soft segment of the PUR until a dominant phase separation was observed. The trifunctional hard segment prevents excessive hydrogen bonding and thus allows for more chain mobility and interaction [14,15]. Generally, PEG is thought to create a highly hydrated surface layer which prevents protein adhesion [16,17], but low-molecular-weight PEG does not have this effect, especially when incorporated into highly crosslinked networks [18,19].

The goal of this study was to investigate a mechanism regulating roles of cell–cell and cell–matrix interactions in activation and apoptosis of macrophages by employing unique phase separation properties of PUR films. Several mechanistic factors were studied by modulating or measuring their activities and production. In order to reduce inflammatory activation of macrophages, an anti-inflammatory peptide, Ac-SDKP, derived from thymosin β -4 in platelets and wound fluid, was used, as this peptide has been identified to decrease macrophage infiltration and TGF- β expression [20]. As inflammatory activation is mediated by the production of reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxide radical ($OH^{\bullet}$), diphenylene iodonium (DPI) (a NADPH oxidase inhibitor) was used to reduce ROS production [21]. Macrophages also secrete tumor necrosis factor (TNF)- α , which induces apoptosis in adherent macrophages and is secreted in high levels when macrophages are cultured on PEG hydrogels [22–25]. The exogenous addition of TNF- α also promotes adhesion of inflammatory cells through a CD11b-dependent mechanism, thereby promoting inflammatory activation of the cells [26]. In contrast, granulocyte–macrophage colony-stimulating factor (GM-CSF) induces

macrophage fusion to form MNGCs, thereby protecting macrophages from apoptosis [2,22,27,28]. Hence, the mechanistic roles of TNF- α and GM-CSF in regulating macrophage apoptosis were studied.

2. Methods and materials

2.1. Preparation of PUR films

2.1.1. Materials—2-Isocyanatoethyl 2,6-diisocyanatoheanoate (lysine tri-isocyanate, LTI) was purchased from Kyowa Hakko USA (New York). Triethylene diamine (TEDA), glycerol, dipropylene glycol (DPG), poly(ethylene glycol) 600 g mol⁻¹ (PEG 600), ϵ -caprolactone and anhydrous dichloroethane (DCE) were purchased from Sigma-Aldrich (St. Louis, MO). Glycolide and D,L-lactide were purchased from Polysciences (Warrington, PA). All other reagents were purchased from Sigma. DPG and PEG 600 were dried over 4 Å sieves before use. TEDA, a well-known tertiary amine polyurethane catalyst with low toxicity, was dissolved in a 33% (w/v) solution with dry DPG. Glycerol was dried under vacuum at 80 °C for 24 h and ϵ -caprolactone was dried over anhydrous magnesium sulfate prior to use.

2.1.2. Polyester synthesis—Trifunctional polyester triols (900 g mol⁻¹) were prepared as described previously [8,29]. Briefly, dried glycerol and ϵ -caprolactone were added to a 100 ml flask containing glycolide, D,L-lactide and stannous octoate. The flask was heated under an argon atmosphere to 135 °C. The reaction was allowed to proceed for 36 h. The hydroxyl (OH) number was determined by titration according to ASTM D4274-99 Method C [8,29]. The molecular weight was verified by gel permeation chromatography (Waters Breeze). The polyester is composed of 60% ϵ -caprolactone, 30% glycolide and 10% D,L-lactide monomers. The polyester was dried under vacuum at 80 °C for 24 h prior to use.

2.1.3. Film production—Thin PUR films were generated by a drop-casting technique. The polyester and PEG components were added to the required catalyst solution in a 5 ml glass tube and dissolved in DCE. The PEG was varied between 0%, 25%, 35% and 50% per mole of the soft segment. The isocyanate was added to a separate glass tube and dissolved in DCE. The two solutions were mixed and vortexed for roughly 30 s, with the final concentration of polymer being 10 wt.%. The polymer solutions were aliquoted onto 12 mm diameter glass coverslips and placed in a dry, nitrogen purged oven at 60 °C overnight.

2.1.4. Non-phase separated alternative substrates—Silicon films (Paso Robles, CA) were punched into 24 wells and used as low adhesion cell substrates. To create high adhesion substrates, silicon films were incubated with 0.2% w/v gelatin with 0.1% glutaraldehyde crosslinking overnight. Films were then washed for times for 45 min per wash with PBS before seeding cells.

2.2. Characterization of PUR films

2.2.1. Phase and fluorescence imaging of phase separated domains—Pure polyester, LTI-PEG and LTI-polyester solvent-cast films were incubated in DMEM for 3 days, stained with Hoechst 33258 nuclear stain (Sigma) and imaged with a Nikon Eclipse Ti inverted fluorescent microscope (Nikon Instruments, Melville, NY).

2.2.2. Contact angle—Static, advancing, and receding contact angles were measured using ultrapure water. Visual readings were taken from a Ramè-Hart Model 100 goniometer ($n = 5$).

2.2.3. Collagen adsorption—PUR films were pre-incubated in DMEM for 24 h and reacted with fluorescein-labeled collagen ($100 \mu\text{g ml}^{-1}$ in PBS, Elastin Products Company, Owensville, MO) overnight. Films were then washed with PBS to remove non-adsorbed collagen and imaged using an inverted fluorescent microscope ($n = 3$). Collagen adsorption was quantified by measuring the average green fluorescence intensity per image using ImageJ (National Institute of Health, Bethesda, MD).

2.2.4. Attenuated total reflectance spectroscopy—Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) measurements were conducted with a Seagull Variable Angle Reflection Accessory (Harrick Scientific) applied to a Tensor 27 FTIR instrument (Bruker Optics, Billerica, MA). A ZnSe hemispherical crystal (Harrick Scientific, Pleasantville, NY) was utilized to obtain time-resolved ATR spectra. For each reaction characterized, spectra were taken every 60 s at 56 scans per spectrum. LTI, polyester or PEG and catalyst were mixed for 1 min and then placed on a sample holder in direct contact with the bottom of the ZnSe crystal. The isocyanate peak (2270 cm^{-1}) was tracked with time. The reaction rates elucidated by this method were utilized to describe network formation. Furthermore, ATRFTIR spectra were taken for each film dry and after being soaked in water for 24 h to analyze the changes in hydrogen bonding. Opus Spectroscopy Software (Bruker) was utilized to fit peaks of interest to determine relative hydrogen bonding.

2.2.5. Thermal analysis—The thermal transitions of the polyurethane films were mapped with a Q200 differential scanning calorimeter (DSC, TA Instruments, New Castle, DE). Films were cut down to 10–15 mg and heated from -80 to 100°C at a rate of $10^\circ\text{C min}^{-1}$, cooled back to -80°C at a rate of $10^\circ\text{C min}^{-1}$ and returned to 100°C at a rate of $10^\circ\text{C min}^{-1}$.

2.3. Cell assays

2.3.1. Cell culture—Human blood-derived monocytes were obtained from Advanced Biotechnologies (Columbia, MD) and differentiated into human blood monocyte-derived macrophages (HBMs). Monocytes at a density of $2 \times 10^6 \text{ cells ml}^{-1}$ were incubated with 10 ml of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 20% fetal calf serum (Invitrogen), 10% human serum (Sigma) and 5 ng ml^{-1} macrophage colony-stimulating factor (Sigma) for 9 days to differentiate into macrophages [30]. PUR films were solvent-cast on glass coverslips of 24-well plate size and were UV sterilized for 1 h prior to cell seeding. Anti-inflammatory peptide Ac-SDKP was obtained from GenScript (Piscataway, NJ). The reactive oxygen species inhibitor diphenyleneiodonium chloride (DPI) was obtained from Sigma. HBMs ($3 \times 10^5 \text{ cells ml}^{-1}$) were cultured on solvent casted films with or without Ac-SDKP ($75 \mu\text{g ml}^{-1}$) or DPI ($4 \mu\text{M}$) for 3 days before endpoint assays. Human umbilical vein endothelial cells (HUVECs; Cell Applications, San Diego, CA) were cultured on PUR films ($8 \times 10^4 \text{ cells ml}^{-1}$) for 3 days in endothelial cell growth medium (Cell Applications), as an indication of naturally adherent cell response to test materials.

2.3.2. Cell attachment, population, and density—After 3 days of culture, cells were washed once with PBS, stained with Hoechst and imaged on an inverted fluorescent microscope. The number of cells was counted per image field ($4 \times 10^4 \mu\text{m}^2$) ($n = 4$). Cell density was determined by dividing each image field into $16,182 \mu\text{m}^2$ grids, and counting the number of grids per image field with more than 4 cells per grid as a representation of high cell density areas ($n = 4$).

2.3.3. Macrophage adhesion marker (CD11b) expression—After 3 days of culture, HBMs were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS for 1 h, blocked with 10% goat serum for 30 min, incubated with FITC-conjugated anti-CD11b antibody (clone number 44, Abcam, Cambridge, MA) for 2 h and imaged with an inverted microscope. CD11b expression was presented as a percentage of CD11b-positive cells (i.e. cells expressing an average fluorescence intensity of >26 relative fluorescence units) relative to the total number of cells per image ($n = 3$).

2.3.4. Cell proliferation—After 3 days of culture, HBMs were incubated with 5-bromo-2'-deoxyuridine (BrdU, Sigma) at 20 μ M for 16 h before staining. The HBMs were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS and DNA was denatured by treatment with hydrochloric acid (1 N for 10 min on ice, 2 N for 10 min at room temperature (RT), then 20 min at 37 °C), which was neutralized with borate buffer (0.1 M, pH 9.0) for 12 min at RT. The HBMs were then blocked with 1% Triton X-100 + 5% normal goat serum for 1 h. Incorporated BrdU in proliferating cells was detected by incubation with rat anti-BrdU antibodies overnight at RT (1:100, Abcam), followed by addition of secondary DyLight594-conjugated goat anti-rat antibodies for 2 h at RT in the dark (1:50, Jackson ImmunoResearch, West Grove, PA). Cell nuclei were counterstained with Hoechst (5 μ g ml⁻¹) in PBS for 15 min at RT. HBMs were imaged under an inverted fluorescence microscope. HBM proliferation was presented as a percent of BrdU-positive cells relative to the total number of cells per image (%) ($n = 3$).

2.3.5. Apoptosis—After 3 days of culture, HBMs were removed from films and analyzed for a percentage of apoptotic cells using APO-BrdU™ TUNEL Assay Kit (Life Technologies, Grand Island, NY) according to the supplier's protocol. A percentage of positively stained apoptotic cells was determined by flow cytometry (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star Inc, Ashland, OR) ($n = 3$).

2.4. Biochemical assays

2.4.1. Matrix metalloproteinase (MMP)-9 activity—After 3 days of culture, the culture medium was removed and concentrated using 10 kDa ultrafiltration filters (Millipore, Billerica, MA). Medium samples (15 μ g of protein per sample) were incubated 1:1 with sample buffer containing 0.15 M Tris-HCl (pH 6.8), 20% glycerol, 0.06% bromophenyl blue and 5% (w/v) sodium dodecyl sulfate. Samples were loaded onto a 7.5% polyacrylamide gel containing 0.1% (w/v) gelatin (Sigma). A 100 V current was applied for 100 min. Each lane was loaded with 5 μ g total protein. Gels were washed four times in dH₂O containing 3.3 vol.% Triton X-100 for a total of 40 min, followed by incubation overnight in 50 mM Tris (pH 7.3) containing 13 mmol of CaCl and 0.05% Brij-35 in dH₂O at 37°C under constant gentle shaking for 18 h. After incubation, gels were fixed in 30% methanol, 10% acetic acid and 60% dH₂O for 1 h before staining with 4 parts of Coomassie brilliant blue R-250 (Sigma) and 1 part of methanol for 20 h. Gels were destained in 25% methanol for 1 h. The gelatinolytic activity of macrophage MMP-9 appeared white on a blue background. The activity of MMP-9 was determined by densitometry using ImageJ ($n = 4$).

2.4.2. Superoxide and phagocytosis assays—After 3 days of culture, HBMs were treated with greenfluorescent *Escherichia coli* particles for 2 h according to the manufacturer's protocol (Vybrant® Phagocytosis assay kit, Life Technologies), stained with dihydroethidium (1 μ g ml⁻¹ DHE) for 15 min [31,32], counterstained with Hoechst and imaged with an inverted fluorescent microscope. The fluorescence intensities of the *E. coli* particles phagocytosed by activated macrophages were measured using a plate reader (Tecan Group, Ltd Männendorf, Switzerland). Phagocytic activity and superoxide production were normalized to cell number measured by Hoechst nuclear stain ($n = 4$) [33,34].

2.4.3. Cytokine secretion—To block CD11b-mediated cell adhesion, HBMs were incubated with anti-CD11b prior to seeding on films [35]. After 3 days of culture, medium samples were collected and analyzed for the released amount of TNF- α and GM-CSF using BD Cytometric Bead Array Flex Sets, a Human Soluble Protein Master Buffer Kit and a FACS Aria Flow Cytometer (BD Biosciences, San Jose, CA) according to the supplier's protocol ($n = 3$).

2.5. Statistical analysis

In all experiments, analytical results are expressed as means $\pm$ standard error of the mean. One-way analysis of variance was used to determine if statistical differences exist between groups. Comparisons of individual sample groups were performed using an unpaired Student's *t*-test. For all experiments, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Contact angle and collagen adsorption

Fig. 1A shows the chemical structure of the monomeric components of the PUR networks. As shown in Fig. 1B, the static contact angle drops from a maximum of $74.2 \pm 3.3^\circ$ with 0% PEG films to a minimum of $44.3 \pm 0.6^\circ$ with 50% PEG films. As the PEG content increases from 0% to 50%, adsorption of collagen, a model protein that is known to affect monocyte differentiation [36–38], increases (Fig. 1C), indicating that polyester domains reduce protein adhesion as compared to low-molecular-weight PEG domains.

3.2. ATR-FTIR, asymmetric reactivity and thermal analysis

Full ATR-FTIR spectra of the films in dry and wet states are shown in Fig. 2A. There is an increase in hydrogen bonding with increasing PEG content due to the hydrophilic nature of PEG, which is observed when dry samples become wet. The free carbonyl peak (1724 cm^{-1}) has a significant shoulder peak, indicating hydrogenbound carbonyl (1710 cm^{-1}). The ratio of these peaks is given in Fig. 2A. There is also an appearance of hydrogen bound urethane (1650 cm^{-1}) when the films are hydrated, and the values are normalized to the $\nu_{\text{as}}\text{C}-\text{C}$ at 2900 cm^{-1} and shown in the table in Fig. 2A.

Chemical kinetic rate constants for the reaction of LTI with PEG and polyester were measured. Interestingly, LTI exhibited biphasic reactivity (Fig. 2B) with PEG and polyester, which is attributed to the structure of LTI. The two primary isocyanates react at a specific rate k_1 , while the secondary isocyanate which reacts at a specific rate k_2 . The ratio of these two specific reaction rates is 2.1 ± 0.4 ; therefore the primary isocyanates react twice as fast as the secondary isocyanate. A kinetic model was utilized to track the progression of each reaction via the conversion of each isocyanate population. Since there is no statistical significance between the reaction of PEG with LTI and the polyester with LTI, there is also no difference between the conversions of isocyanate with changing percentage of PEG (Fig. 2C). The network formation proceeds at the same rate; therefore, differences in polymerization should be minimal.

DSC scans with corresponding glass transitions are shown in Fig. 2D. The T_g decreases slightly with 35% and 50% PEG. Pure PEG with a molecular weight of 600 Da has a T_g of -35°C , while the polyester has a T_g of -41.7°C [39,40]. The small shift in T_g indicates marginal phase mixing of the soft and hard segments, regardless of the incorporation of PEG.

3.3. Cell adhesion, population and density

When incubated with culture medium, the PUR films with greater than 20% PEG content underwent phase separation into macrodomains. Polyester domains were stained with Hoechst, as evidenced by blue fluorescence, while no fluorescence was observed in PEG domains (Fig. 3A and B). Films with 0–20% PEG in the soft segment did not separate into macrodomains (Fig. 3B, “0% PEG”) and absorbed Hoechst stain uniformly throughout the film (Fig. 3B). When HBMs were cultured on the PUR films, they preferentially adhered to the non-fluorescent, PEG-containing domains (Fig. 3B, 35% and 50% PEG). As the PEG% in the films increased, the number of HBMs adhered to these films (Fig. 3C), as well as the number of “highly dense” areas (defined as any 16,182 μm^2 grid with more than four cells) increased, because HBMs congregated on the PEG domains (Fig. 3D).

Adhesion of HUVECs is required for their survival and functions, whereas HBMs undergo transition from non-adherent to adherent only when they are activated. Therefore, HUVECs were cultured on PUR films to see whether the selective adhesion to low-molecular-weight PEG is not specific to HBMs. In this way, the current design of PUR films can be proven to allow for cell attachment of naturally adherent cells used for tissue engineering applications (Fig. 3E) [41]. HUVECs fully attached and spread out on 50% PEG films (Fig. 3E, far right), while no spreading of HUVECs was observed on 0% PEG films (Fig. 3E, far left), indicating attachment of both cell types (HBMs and HUVECs) was promoted as the PEG content increased.

3.4. HBM adhesion, proliferation and apoptosis

Expression of CD11b, a macrophage adhesion molecule [42], also increased as the PEG content increased (Fig. 4A). The anti-inflammatory peptide Ac-SDKP and the ROS inhibitor DPI did not significantly alter CD11b expression (Fig. 4B). Macrophage proliferation, as measured by BrdU expression, was not significantly influenced by the changes (0–50%) in PEG content in the PUR films (Fig. 4C). Interestingly, HBM apoptosis was promoted on 50% PEG films compared to 0% PEG films, indicating adhesion-mediated spontaneous apoptosis of HBMs (Fig. 4D) [3]. When the anti-inflammatory Ac-SDKP peptide or the ROS inhibitor DPI was added to low-adherent HBMs on 0% PEG films, apoptosis was promoted. However, the treatments reduced apoptosis in adherent HBMs on the 50% PEG films, indicating that inflammatory activation or ROS production leads to apoptosis of adherent HBMs but is required for survival of low-adherent HBMs.

3.5. MMP-9 activation, phagocytosis and intracellular ROS production

MMP-9 activation, phagocytosis and ROS production are indicators of monocyte/macrophage activation [43,44]. Secreted MMP-9 activity, phagocytic activity and intracellular superoxide production were higher in low-adherent HBMs on 0% PEG PUR films compared to adherent HBMs on 50% PEG PUR films (Fig. 5A–C), likely due to the low level of apoptosis (Fig. 4D). When Ac-SDKP or DPI was added to low-adherent HBMs on 0% PEG PUR films, MMP-9 activation was reduced significantly, but it was increased significantly in adherent HBMs on 50% PEG PUR films (Fig. 5A), indicating a causative role of apoptosis (Fig. 4D) in reducing MMP-9 activation. However, when Ac-SDKP was added to adherent HBMs on 50% PEG PUR films, phagocytosis and superoxide production were both significantly reduced compared to HBMs in the absence of AC-SDKP (Fig. 5B and C). These results suggest that, when HBMs adhered to 50% PEG PUR films, ROS production and phagocytosis were directly down-regulated by the anti-inflammatory activity of AC-SDKP, whereas MMP-9 activation was promoted as apoptosis decreased in the presence of AC-SDKP.

3.6. Roles of cooperative actions of cell–cell and cell–material interactions in HBM apoptosis

As the number of adherent HBMs increased, the cell density also increased (Fig. 1C and D). Therefore, to elucidate the competitive or cooperative actions between cell–cell and cell–material interactions in regulating apoptosis, HBMs were cultured on low-adhesion, uncoated silicon and high-adhesion, gelatin-coated silicon substrates at either a low or high cell density. CD11b expression was promoted when the substrate was changed from low to high adhesion but was unaffected by the change in cell density (Fig. 6A). In contrast, HBM apoptosis increased remarkably when the cell density increased in combination with increased cell adhesion (Fig. 6B). Hence, the highest percent of apoptotic cells was seen on adhesive substrates with high cell density. These results indicate that HBM apoptosis is regulated not only by CD11b-mediated adhesion but also by synergistic cooperation with cell–cell interactions, likely due to the efficient propagation of apoptotic signaling to neighbor cells.

3.7. Roles of cytokines in cooperative cell–cell and cell–matrix interaction-mediated HBM apoptosis

As HBM attachment was promoted most on the adhesive 50% PEG film among the test substrates, this substrate was used for further investigation of a mechanism-governing effect of cell–cell (cell density) and cell–material (adhesion) interactions on HBM apoptosis and cytokine secretion. To reduce HBM adhesion to levels comparable to those observed on 0% PEG films, antibodies to the cell adhesion marker CD11b were incubated with HBMs prior to seeding. HBM apoptosis decreased when adhesion was blocked using CD11b antibodies compared to the non-blocking condition (Fig. 7A), validating the use of CD11b antibody to reduce adhesion similar to the low-adhesive 0% PEG PUR films. Cell density also influenced apoptosis, as evidenced by the increased percentage of apoptotic cells when HBMs were cultured at high cell density compared to low cell density. Ac-SDKP and DPI increased apoptosis of low-adherent HBMs at low cell density (Fig. 7A) in a manner similar to Ac-SDKP and DPI treatment on 0% PEG (Fig. 4D). At the high cell density with normal adhesion, however, Ac-SDKP and DPI decreased apoptosis, further confirming the difference between high adhesion with high cell density and low adhesion with low cell density of HBM in apoptotic response to Ac-SDKP or DPI treatment (Fig. 4D).

Secretion of GM-CSF was not altered significantly when HBMs were cultured on 0% PEG compared to those on 50% PEG films, nor did it change significantly with Ac-SDKP or DPI treatment, indicating that GM-CSF was not involved in activation or apoptosis of HBMs (Fig. 7B) [2,22,27,28]. Secretion of an apoptotic cytokine, TNF- α , in response to changes in adhesiveness, cell density and treatment of Ac-SDKP or DPI (Fig. 7C) showed similar trends to those of apoptosis: TNF- α was secreted most from HBMs at high adhesion with high density; even at high adhesion, it was secreted more from HBMs with high density compared to low density; in the presence of Ac-SDKP, its secretion was promoted significantly at low adhesion with low density but reduced significantly at high adhesion with high density; and treatment of DPI reduced its secretion significantly at high adhesion with high density. These results suggest a potential mechanistic role of TNF- α in promoting spontaneous apoptosis when HBM adhesion and density increases on a biomaterials implant.

4. Discussion

Monocytes play a major role in initiating, maintaining and resolving host inflammatory responses to biomaterial implants by differentiating into macrophages upon adhesion to implants and/or by releasing cytokines. In the absence of inflammatory activation, more monocyte precursors are produced from the marrow than what are needed to replace normal

tissue macrophages [45]. During inflammatory responses to biomaterial implants, a dramatic up-regulation of monocyte survival and differentiation may be required. Thus, the processes involved in regulating removal and survival of monocytes/macrophages are critical to population control for a proper host response to implants. Otherwise, most biomaterial implants would be impaired, as activated monocytes/macrophages can degrade implants through phagocytosis and ROS production. Monocyte apoptosis can be prevented by activation stimuli, such as endotoxin treatment [46]. In the absence of activation stimuli, monocytes undergo spontaneous apoptosis [45]. In agreement with these previous observations, we found that low adherent HBMs underwent apoptosis when their activation was reduced by treatment of the antioxidant DPI or the anti-inflammatory Ac-SDKP peptide (Fig. 7A). Sharma et al. [47] also reported that Ac-SDKP discouraged inflammation by inhibiting the differentiation, activation, migration and/or TNF- α secretion of macrophages. In our study, at low density on low-adhesion materials, the addition of Ac-SDKP reduced the differentiation and activation of macrophages, thereby preventing spontaneous apoptosis. However, when HBMs were adherent at high cell density, their spontaneous apoptosis decreased significantly through a loss of activation in response to Ac-SDKP or DPI, suggesting a new mechanism inducing selective apoptosis of activated HBMs that are adherent to biomaterials at high cell density. Our results indicate that this spontaneous apoptosis of HBMs is induced by CD11b-mediated adhesion, followed by production of an apoptotic cytokine (TNF- α) [22,23,48], which is stimulated by ROS. Anti-inflammatory Ac-SDKP was proven to prevent this mechanism. A previous study has reported a causative role of autocrine TNF- α signaling in promoting CD11b-mediated adhesion of macrophages [26]. In contrast, the current study demonstrates the converse relationship, in which an increase in CD11b-mediated adhesion of macrophages promotes their TNF- α secretion. This suggested mechanism provides insight into a new approach to regulate monocyte/macrophage interactions with biomaterials for successful implants by altering the cell-material and cell-cell interactions. In Figs. 4B and 6A, more than 50% of macrophages expressed CD11b in all the test conditions, indicating important roles of CD11b in a variety of basic macrophage activities (e.g. adhesion and phagocytosis) [49]. Interestingly, exogenous stimulation such as ROS, anti-inflammatory treatment and alternation of cell density resulted in small changes in CD11b expression, but these small changes were enough to regulate macrophage apoptosis.

The inclusion of low-molecular PEG (600 Da) into the PUR networks lowered the contact angle, as expected. The increase in collagen adsorption matches previous studies where polar, hydrophilic soft segments increased protein adsorption [37]. It is widely understood that there is a direct correlation between protein adsorption and cellular attachment [50]. Based on the reactivity and DSC analysis, there appears to be very little difference in the polymerization mechanism or final polymer network to alter the protein adsorption characteristics. Thus, the differences must be largely due to changes in the surface chemistry with the inclusion of low-molecular-weight PEG. The behavior of PEG in PUR materials is dependent on molecular weight and chain mobility [17,18,51,52]. When higher-molecular-weight PEG (>1000 Da) is incorporated into PUR elastomers, there is a nominal decrease in protein and cellular adsorption [17,18]. Conversely, others have seen that at molecular weights closer to 600 Da this reduction in protein adsorption disappears [18]. The phase separation observed when PEG content in the soft segment is 35–50% is dominated by PEG-rich domains with polyester-rich islands. This correlates well with the commonly understood mechanism of hydrophilic chain migration in aqueous environments [16]. The polyester-rich islands that remain exposed are locked into the network, unable to migrate down. Visualization of these phase domains was possible by Hoechst staining as this dye binds to all polymers with a negative charge, including not only DNA but also negatively charged synthetic polymers. A previous study has demonstrated that phase separation can be visualized by optical imaging [53,54]. The current study suggests a new way to improve

fluorescence imaging of phase domains through staining driven by a specific interaction between fluorescence dyes and domains.

The PUR system provided a unique template to regulate the cell–material (HBM adhesion) and cell–cell (HBM density) interactions of HBMs *in vitro*, thereby enabling study of the apoptotic and activation mechanisms. While it was an unexpected consequence of phase separation, HBMs preferentially adhered to PEG domains, resulting in a high cell number and cell density on 50% PEG PUR films compared to 0% PEG PUR films. These trends conflict with previous studies which demonstrated increased macrophage adhesion on more hydrophobic surfaces [1,2,22]. However, the contact angle of the hydrophilic materials in the previous studies was much lower (28°) than the most hydrophilic material in the current study (44° on 50% PEG films). The most hydrophilic material used in this study (50% PEG films) can still promote cell attachment, as demonstrated by the attachment of HBMs and HUVECs. Since both cell–cell and cell–material interactions increased simultaneously on 50% PEG films, model material films with well-accepted cell-adhesion properties and antibodies to block macrophage adhesion integrin CD11b were used to confirm a cooperative role of these two types of interactions in inducing spontaneous HBM apoptosis.

As adhesion increased with the incorporation of PEG, apoptosis increased while HBM activation (i.e. phagocytic activity, superoxide production and MMP-9 activity) decreased. Apoptosis is a useful mechanism for removing and limiting macrophages on an implanted device [55]. Inducing apoptosis without activating macrophages to secrete ROS and MMPs or phagocytose materials, as demonstrated by culturing HBMs on 50% PEG films, is a promising tactic for minimizing the inflammatory response to biomaterials. Previous studies found similar macrophage activation and apoptotic responses with increased substrate hydrophilicity [1,2,22]. However, the current study is the first to identify that macrophage apoptosis can be promoted by a synergistic cooperation between cell adhesion (cell–matrix interaction) and high cell density (cell–cell interaction). This material design provides a useful template to selectively promote an interaction with desirable cells to an implant, such as endothelial cells, while simultaneously reducing inflammatory activation, which is desirable for tissue engineered approaches.

5. Conclusion

As summarized in Fig. 8, phase separation between low-molecular-weight PEG (600 Da) and polyester domains on the PUR surface provided a novel means to elucidate an unknown mechanism driving spontaneous apoptosis of HBMs. As HBMs preferentially adhered to the PEG domains, apoptosis was promoted, while inflammatory activation as measured by phagocytosis, ROS and MMP-9 production decreased compared to films without PEG, suggesting a potential material design to allow for cell adhesion without stimulating excessive inflammatory activation. This study also identified a mechanism driving the CD11b adhesion-based spontaneous apoptosis of macrophages through TNF- α signaling, which can be down-regulated by ROS inhibitor DPI and anti-inflammatory peptide Ac-SDKP.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1, 3 and 8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.actbio.2012.10.038>.

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Fig. 1.

Chemical structures and surface adsorption of PUR films. (A) Monomer structures of the PUR network formed by the reaction of an isocyanate group on LTI and a hydroxyl group on either the polyol or PEG. (B) Static, advancing and receding water contact angle on PUR films as measured by contact angle goniometry. (C) Adsorption of green fluorescently labeled collagen after 24 h incubation on PUR films. Scale bar = 100 μ m. Collagen adsorption was quantified by calculating the average green fluorescence intensity per image (graph). (B–D) * p < 0.05 vs. 0% PEG-containing PUR films.

Fig. 2. Characterization of PUR films. (A) ATR-FTIR spectral profiles of dry (top) and wet (middle) PUR films with increasing PEG content and their quantified values (bottom table) for hydrogen bonding and PEG migration. (B) Second-order reaction rate plots (top graph) for the LTI-PEG and LTI-polyester reactions (dual reactivity is shown as k_1 and k_2) and quantified reaction rates (bottom table) for each component of the PUR films along with the ratio of k_1/k_2 . (C) Theoretical conversion of isocyanate population in 0% (top) and 50% (bottom) PEG-containing PUR films based on chemical kinetic reaction rates. (D) DSC scans (top) and glass transition temperature (bottom table) of PUR films with increasing PEG content.

Fig. 3.

Phase separation and cell attachment to PUR films. (A, B) Fluorescence images of phase separation between polyester (bright blue) and PEG (no fluorescence) in PUR films after incubation with Hoechst stain, and phase contrast images of HBMs cultured for 3 days on PUR films with 0%, 20%, 35% or 50% PEG in the soft segment (scale bar = 40 μm); (C) cell number and (D) cell density of HBM per image field. * $p < 0.05$ vs. 0% PEG-containing PUR films; (E) HUVEC attachment on 0% to 50% PEG-containing PUR films (scale bar = 100 μm).

Fig. 4. HBM adhesion, proliferation, and apoptosis on PUR films. Expression of adhesion glycoprotein CD11b in HBMs (A) on 0%, 20%, 35% and 50% PEG-PUR films, and (B) on 0 and 50% PEG films in the presence of Ac-SDKP (anti-inflammatory peptides) or DPI (ROS inhibitor); (C) proliferating HBMs (%) as measured by BrdU on 0%, 20%, 35% and 50% PEG films; (D) apoptotic HBMs (%) measured by Apo-BrdU TUNEL assay on 0 and 50% PEG films, with or without Ac-SDKP or DPI. (A, C) $*p < 0.05$ vs. 0% PEG; (B, D) $*p < 0.05$ vs. no peptide on same PEG-content material, $\dagger p < 0.05$ between the groups connected by lines.

Fig. 5. Inflammatory activation on PUR films. (A) Activity of MMP-9 secreted by HBMs on 0% and 50% PEG films with or without Ac-SDKP or DPI, as measured by zymography; (B) phagocytic activity of HBMs with or without Ac-SDKP on 0%, 20%, 35% or 50% PEG films as measured by Vybrant phagocytosis assay; (C) intracellular superoxide production of HBMs as measured by staining with dihydroethidium (DHE). (A–C) $*p < 0.05$ vs. 0% PEG with same treatment (no peptide, Ac-SDKP or DPI); $^{\dagger}p < 0.05$ vs. no peptide on same PEG-containing substrates.

Fig. 6. Roles of cell-cell and cell-material interactions in apoptosis. (A) Expression of CD11b adhesion marker and (B) apoptosis of HBMs cultured at low (2×10^5 cells ml^{-1}) and high (4×10^5 cells ml^{-1}) cell density on non-adherent (silicon) or adherent (silicon with gelatin coating) films. * $p < 0.05$ compared to low cell density on the same substrate; † $p < 0.05$ between the groups connected by lines.

Fig. 7. Roles of cytokines in cell-cell and cell-material interaction-mediated apoptosis on 50% PEG-containing PUR films. (A) Percent apoptotic HBMs and secretion of (B) GM-CSF and (C) TNF- α of HBMs cultured at low (2×10^5 cells ml^{-1}) and high (4×10^5 cells ml^{-1}) cell density on 50% PEG films with or without Ac-SDKP or DPI treatment. LD = low cell density, HD = high cell density. * $p < 0.05$ between the groups connected by lines; † $p < 0.05$ vs. blocked adhesion with same cell density and treatment.

PUR biomaterial (unique design)

Fig. 8.

Suggested mechanism driving HBM apoptosis PUR films. Phase separation between PEG and polyester domains on PUR surface provided a novel means to elucidate an unknown mechanism driving apoptosis of HBMs. As HBMs preferentially adhered on phase domains of low-molecular-weight PEG, the local cell density increased only in the PEG domains. A dominant causative role of cell–matrix interaction in spontaneous apoptosis of HBMs was mediated through CD11b expression, which was synergized with increased cell–cell interactions and increased PEG content in PUR films. As TNF- α secretion increased, CD11b adhesion-mediated apoptosis was promoted. Inhibition of ROS production and inflammatory activation reduced TNF- α secretion and consequent apoptosis, indicating their upstream mechanistic roles in regulating spontaneous apoptosis of HBMs.