Protein-mediated macrophage adhesion and activation on biomaterials: a model for modulating cell behavior

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The elucidation of proteins involved in biomaterial-modulated macrophage behavior is critical for the improvement of material performance and the initial exploration of material design capable of manipulating macrophage function for tissue engineering. In this paper, several *in vitro* and *in vivo* techniques are presented to demonstrate means of delineating a part of the complex molecular mechanisms involved in the interaction between biomaterial and macrophage adhesion and phenotypic development. The following conclusions were reached: (1) using radioimmunoassay, complement component C3 was found to be critical in mediating human macrophage adhesion on polyurethanes. (2) The presence of a diphenolic antioxidant additive in polyurethanes increased the propensity for complement upregulation but did not affect adherent macrophage density. (3) The subcutaneous cage-implant system was utilized to delineate interleukin-4 participation in the fusion of adherent macrophages to form foreign body giant cells *in vivo* in mice. The injection of purified interleukin-4 neutralizing antibody into the implanted cages significantly decreased the giant cell density; conversely, the giant cell density was significantly increased by the injection of recombinant interleukin-4 when compared with the controls. (4) The RGD and PHSRN amino acid sequences of the central cell binding domain and the PRRARV sequence of the C-terminal heparin binding domain of human plasma fibronectin were utilized to study the structure-functional relationship of protein in mediating macrophage behavior. Polyethyleneglycol-based networks grafted with the RGD-containing peptide supported higher adherent human macrophage density than surfaces grafted with other peptides. The formation of foreign body giant cell was highly dependent on the relative orientation between PHSRN and RGD domains located in a single peptide.

Nomenclature

FBGC	foreign body giant cell
C3	complement component 3
PEUU	polyetherurethane urea
II-4	interleukin-4
RGD	tripeptide arginine–glycine–aspartic acid
PHSRN	pentapeptide proline–histidine–serine–arginine–asparagine
PRRARV	hexapeptide proline–arginine–arginine–alanine–arginine–valine

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1. Introduction

The host inflammatory reaction is a normal response to injury and the presence of foreign objects. The magnitude and duration of the inflammatory process has a direct impact on the stability and compatibility of biomaterials, hence affecting the efficacy of biomedical devices [1, 2]. Macrophage is a central cell type in directing host immune and inflammatory process; thus, its response to biomaterials is of extreme importance in understanding material–host interrelationships. Several characteristic macrophage functions are identified as critical events in the interaction with biomaterials. Macrophages recognize the adsorbed proteins on the

biomaterial surface and may adhere onto the surface via several adhesion ligand–receptor superfamilies [3]. The process of adherent macrophage activation and fusion to form multinucleated foreign body giant cells (FBGC) is unique to the macrophage phenotype. FBGCs have been demonstrated on biomaterials and the rate of material degradation underneath FBGCs has been shown to be markedly increased [4]. Activated macrophages may further release factors to modulate the function of other cell type in the inflammatory milieu [5].

To elucidate the exact mechanisms of which material surface and bulk properties mediate macrophage behavior, current research efforts focus on the interplay between material parameters (i.e. chemistry, hydrophobicity, mechanical properties) and observed macrophage functions (i.e. adhesion, FBGC formation, cytokine and growth factor release) [3, 6–8]. Such investigation enables biomaterial scientists to exploit the design of materials that would lead to favorable macrophage–surface interaction. For example, it may be possible to harness some of the beneficial functions of the macrophage to promote biocompatibility, such as the expression of angiogenic and other growth factors, while avoiding some of their detrimental behavior, including fusion to form FBGCs. Controlled macrophage function would have a wide therapeutic potential, such as regulating tumor cells, mediating angiogenesis, or inducing the release of specific cytokines and growth factors to direct host healing, immune, and inflammatory responses. Before the ability to control specific macrophage behavior via biomaterials can be realized, a more detailed understanding of the interplay between material-bound ligands and receptors on the cell surface must be obtained, and in doing so the challenge of eliciting a specific cellular function in the presence of redundancies that exist between receptors and target proteins must be addressed.

The development of quantitative methodologies are crucial in the elucidation of macrophage–material interaction. *In vitro* protocol allows the investigators to address specific cellular event under a controlled environment; however, concurrent investigation under an appropriate *in vivo* system is necessary to confirm and to re-evaluate *in vitro* observation. In this paper, several *in vitro* and *in vivo* techniques are presented to demonstrate means of delineating a part of the complex molecular mechanisms involved in the interaction between biomaterials and macrophages. A clear understanding of material-mediated macrophage function enables the initial exploration of material design capable of modulating cell behavior.

2. Adsorbed proteins mediate macrophage adhesion

The complement cascade consists of various blood proteins that, upon activation by the presence of foreign objects, mediates host immune and inflammatory response. The activation of the complement cascade by biomaterials occurs primarily through the alternative pathway with complement component C3 being a critical protein. C3 has been shown to adsorb onto biomaterial surfaces in a fragmented form called C3b. Adsorbed C3b further complexes with complement factor B or factor H in a series of events resulting in the formation of C3b or iC3b, respectively. Adsorbed C3b or iC3b are ligands for macrophages via CD35 or CD11b/CD18 and CD11c/CD18 cell-membrane receptors, respectively [9–13].

A type of medical-grade polyetherurethane urea (PEUU), with or without a diphenolic antioxidant additive [14, 15], was used as a model to investigate the role of critical complement proteins in mediating macrophage adhesion as a function of material composition. To determine the adsorption of adhesion-promoting proteins and various complement components, a modified radioimmunoassay based on protein G binding to the primary antibody was utilized. This method is capable of detecting multiple surface-bound proteins from a multicomponent system [8, 9, 16]. Briefly, PEUUs were exposed to 20% human serum supplemented with RPMI culture medium for 1 h. The treated samples were rinsed with phosphate-buffered saline and incubated in $20\,\mu g\,ml^{-1}$ protein G for 1 h at 37 °C. The samples were then rinsed and incubated with anti-human antibodies against complement component C3, factor B, factor H, and fibronectin. The samples were rinsed, incubated with I^{125}-conjugated protein G for 1 h at 37 °C, and the radioactivity was quantified using a 1261 LKB Wallac Multigamma gamma-counter normalized to the sample surface area. No differences ($P > 0.05$) in radioactivity were observed for C3, Factor H, or fibronectin on PEUUs with or without the antioxidant (Table I) indicating that the presence of antioxidant in PEUUs did not affect the amount of adsorbed C3, factor H, or fibronectin. However, a higher level of factor B ($P < 0.05$) adsorption was found on PEUUs containing the antioxidant when compared with PEUUs without the antioxidant. These results indicate that the presence of antioxidant in PEUUs significantly increased factor B adsorption, suggesting a higher propensity for further complement activation.

To determine the relative importance of the complement cascade in mediating macrophage adhesion on

TABLE I Complement protein adsorption on PEUUs

Material	C3	Factor B	Factor H	Fibronectin
PEUU	140 ± 38	6 ± 9	28 ± 20	16 ± 15
PEUU + antioxidant	152 ± 52	28 ± 8[a]	54 ± 19	11 ± 8

Units, radioactivity c.p.m. minus no antibody control per mm^2, mean $\pm$ SEM, $n = 4$.
[a] $P < 0.05$ versus respective PEUU value.

biomaterials, a critical complement component, namely C3, was depleted from healthy human serum using anti-C3 antibody affinity column. As a comparison, fibronectin was depleted from the serum using gelatin sepharose chromatography. All protein depletion was confirmed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot with polyclonal antibody against C3 or fibronectin [9]. Human blood monocytes were isolated from healthy adult donors per procedures previously described [17] and incubated with PEUUs in the presence of 20% normal non-depleted, C3-depleted, or fibronectin-depleted serum in RPMI for 2 h under 5% CO_2 at 37 °C. The samples were rinsed and fixed; adherent macrophages were quantified using a computerized video analysis system (JAVA Jandel Scientific) coupled to an Olympus BH-2 light microscope. The results showed that the macrophage density on PEUUs with or without the antioxidant was significantly lower ($P < 0.05$) when C3 was depleted from the culture medium, as compared to the macrophage density when non-depleted serum medium was used (Table II). No differences in macrophage density were found for both PEUUs when normal non-depleted or fibronectin-depleted serum were used. These results indicate that the complement system, specifically the presence of C3, is critical in mediating macrophage adhesion on PEUUs. In concert with the above protein adsorption study, these results indicate the following. The presence of adsorbed factor B was used to assess the propensity for complement up-regulation. Factor B may complex with C3b resulting in the formation of more C3b which are potential adhesion ligands for macrophages. Although the presence of antioxidant in PEUUs increased factor B adsorption when compared with PEUUs without the antioxidant, this increase did not result in an increase in the adherent macrophage density. It is clear that the quantification of adsorbed proteins on biomaterials is important; however, the effect of this protein layer on cell behavior must also be investigated in tandem to obtain a more complete picture of the interrelationship among material properties, protein adsorption, and cellular events.

3. Soluble factors mediate the fusion of adherent macrophages to form foreign body giant cells

A unique macrophage phenotypic development pertinent to biomaterials is the formation of multinucleated FBGCs from the fusion of adherent macrophages. The formation of FBGCs has a direct correlation to the degradation of biomaterials [4]. Previous *in vitro* results demonstrated that the pleiotropic lymphokine interleukin-4 (IL-4) plays a critical role in mediating the fusion of adherent macrophages to form FBGCs on modified tissue-culture polystyrene [17]. To extend this finding into an *in vivo* environment on biomedically-relevant materials, the subcutaneous cage-implantation system was utilized [18]. PEUU films without the diphenolic antioxidant additive were placed inside a cylindrical cage (3.5 cm long and 1 cm in diameter) constructed from medical-grade stainless-steel wire mesh. The samples were gas sterilized using ethylene oxide and implanted subcutaneously at the back of 8-week-old, female BALB/c mice (Jackson, Bar Harbor, ME). Goat antimouse IL-4 neutralizing antibody (R & D) at various concentrations was injected directly into the cage. The injection of goat immunoglobulin G (IgG; R & D) or phosphate-buffered saline (PBS) (Sigma) were used as negative controls and mouse IL-4 (R & D) was utilized as positive controls. The injections were given every other day post-implantation and the PEUU samples were retrieved after 10 days of implantation. Adherent cell density and size were quantified as described above.

The results showed that a concentration of 250 μg ml^{-1} of anti-IL-4 antibody injected directly into the cage significantly decreased ($P < 0.01$) FBGC density on PEUUs when compared with the IgG- and PBS-injection controls (Table III). Conversely, the presence of additional IL - 4 inside the cage significantly ($P < 0.01$) increased FBGC density on PEUUs when compared with the controls. Comparable average FBGC size and macrophage density were observed for all groups. These results confirmed the *in vitro* finding that IL-4 plays a critical role in the formation of FBGCs on PEUUs *in vivo*.

4. Design polymer networks grafted with protein-derived oligopeptides to control macrophage behavior

The challenge of eliciting specific macrophage function by utilizing biomaterials lies within the host foreign body response which might overcome the bioactive functionalities in the material designed to mediate macrophage behavior. Furthermore, most macrophage-activating proteins may elicit several macrophage functions; thus a clear understanding of the structure–functional relationship between target proteins and cell membrane receptors is crucial. Based on these design requirements, a polymer network based on the terpolymerization of monomethoxy polyethyleneglycol monoacrylate, acrylic acid, and trimethylolpropane triacrylate was designed and synthesized [19, 20]. The polymer networks were

TABLE II Adherent macrophage density on PEUUs in the presence of various protein-depleted culture media

Material	Normal non-depleted medium	C3-depleted medium	Fibronectin-depleted medium
PEUU	680 ± 160	150 ± 70[a]	660 ± 160
PEUU + antioxidant	640 ± 210	170 ± 90[a]	610 ± 210

Units, cells mm^{-2}, $n = 4$, mean ± SEM.
[a] $P < 0.05$ versus respective normal non-depleted medium controls.

TABLE III Effects of II-4 on FBGC formation on PEUU *in vivo*

Treatment	Macrophage density	FBGC density	Average FBGC size
IL-4	802 ± 139	61 ± 4*	2.9 ± 0.9
IL-4 antibody	530 ± 202	14 ± 3*	2.1 ± 1.8
IgG control	646 ± 131	44 ± 10	2.8 ± 0.5
PBS control	731 ± 211	43 ± 11	3.6 ± 1.1

Density in $\times$ 100 cells cm^{-2}, cell size in $\times 10^{-3}$ cm^2, mean $\pm$ SEM, $n = 4$. Injections given every other day at 100 ng of murine IL-4, 250 μg of goat anti-mouse II-4 antibody or goat IgG per 1 ml of PBS.
[a] $P < 0.01$ versus respective control values.

incubated with a model macrophage cell line, murine IC-21 (ATCC) [21]. Human dermal fibroblasts and human umbilical vein endothelial cells (Gibco) were utilized as comparisons between cell types. For each cell type, 10^6 cells were suspended in 1 ml of culture medium supplemented with 20% serum. A very low level of cell adhesion (< 8 cells mm^{-2}) was observed for all cell types up to 10 days of culture even in the presence of serum- and cellularly derived adhesion proteins. The material was functionalized [19, 20] to covalently graft bioactive factors designed to mediate cell adhesion and function. These bioactive factors consisted of several peptides derived from both extracellular matrix adhesion proteins and macrophage-active proteins that are normally soluble. The candidate peptides examined corresponded to residues 63 to 77 of complement component C3a ($C3a^{(63–77)}$) [22], residues 178 to 207 of interleukin-1β ($IL\text{-}1\beta^{(178–207)}$) [23], residues 1615 to 1624 containing the tripeptide arginine glycine-aspartic acid (RGD) sequence from the central cell-binding domain of fibronectin of fibronectin ($FN^{(1615–1624)}$) [24], endothelial-macrophage activating polypeptide II [25], complement component C5a inhibitory sequence [26], and macrophage inhibitory peptide [27]; YRGDG pentapeptide [28] was used as a control and networks without grafted peptides were negative controls. Peptides were either purchased commercially (Bachem) or synthesized using an automated peptide synthesizer (9050 Pep Plus Synthesizer, Millipore).

When compared amongst the samples, $IL\text{-}1\beta^{(178–207)}$- and $C3a^{(63–77)}$-grafted networks supported higher IC-21 macrophage densities (40 ± 10 and 50 ± 10 cells mm^{-2}, $n = 4$, mean $\pm$ SEM, respectively), $C3a^{(63–77)}$- and $FN^{(1615–1624)}$-grafted samples had higher fibroblast densities (30 ± 5 and 70 ± 20 cells mm^{-2}, $n = 4$, mean $\pm$ SEM, respectively), $FN^{(1615–1624)}$- and YRGDG-grafted substrates had higher endothelial cell densities (both 20 ± 5 cells/mm^{-2}, $n = 4$, mean $\pm$ SEM) up to 98 h. For all samples, more than 95% of adherent cells showed extensive cytoplasmic spreading and pseudopodial extension after 24 h of culture. The specificity of cell receptors and immobilized peptides was determined by competitive studies. Cells were preincubated with free peptides for 30 min (1×10^6 cells ml^{-1} with 0.25 or 0.0025 mg ml^{-1} of soluble peptides): IC-21 macrophages with $IL\text{-}1\beta^{(178–207)}$ or $C3a^{(63–77)}$, fibroblasts with $C3a^{(63–77)}$ or $FN^{(1615–1624)}$, endothelial cells with $FN^{(1615–1624)}$ or YRGDG. The treated cells were exposed to surfaces grafted with respective peptides and cultured. Nominal adherent cells (< 5 cells mm^{-2}, $n = 4$, mean $\pm$ SEM) were observed for all cell types up to 168 h independent of the free peptide concentration used in the preincubation. The non-adherent cells and free peptides were removed, the networks were rinsed, and the same sample was incubated with fresh, untreated cells. After 2 h of culture, adherent cell densities increased significantly and were restored to values comparable to the above cell adhesion results. The adherent cell densities increased steadily with increasing time. Adherent IC-21 macrophage and fibroblast densities were confluence after 168 h (approximately 700 and 300 cells mm^{-2}, $n = 4$, mean $\pm$ SEM, respectively). These results confirmed the specificity between cell receptors and grafted peptides in mediating cell adhesion.

The above results showed that peptides immobilized on the polyethyleneglycol-based network modulate the adhesion of distinct cell type in a receptor-specific manner. The grafted peptides and adherent cells are utilized for further mechanistic studies and cellular manipulations. To probe the structure-functional requirement of cellular activation modulated by receptor-protein complexation, a series of biomimetic oligopeptides were designed based on the known bioactive regions of fibronectin [29]. Oligopeptides containing the RGD and pentapeptide proline–histidine–serine–arginine–asparagine (PHSRN) sequences of the central cell binding domain [30] and the hexapeptide proline–arginine–arginine–alanine–arginine–valine (PRRARV) sequence of the C-terminal heparin binding domain [31] of fibronectin were designed and synthesized with an automated peptide synthesizer described above. Each synthetic peptide contains one or two functional domain(s) at both possible orientations (i.e. peptides containing both the RGD sequence and the PHSRN sequence from C-terminus to N-terminus or vice versa) with interpositional hex-Gly sequence. Tri-Gly sequence was used as a spacer at the C-terminus for all peptides. Polyethyleneglycol-based networks, as described above, were employed as substrates for peptide grafting and the samples were incubated with blood-derived human macrophages isolated per procedures described previously [17] in the presence of autologous serum. The networks grafted with GGGRGDG supported comparable adherent macrophage density as the tissue-culture polystyrene reference material (378 ± 22 and 399 ± 16 cells mm^{-2}, $n = 6$, mean $\pm$ SEM, respectively) and both surfaces showed significantly higher ($P < 0.10$) adherent macrophage density than networks grafted with other peptides. Using monoclonal neutralizing anti-integrin β1 antibodies (60 μg ml^{-1}, Boehringer Ingelheim), nominal macrophage adhesion

($< 6\,\text{cells}\,\text{mm}^{-2}$) were found on all peptide-grafted surfaces. These data indicate that the grafted peptides mediated macrophage adhesion primarily through the complexation between cell membrane integrin β_1-containing receptors and the grafted peptides. Furthermore, under cell culture conditions conducive to FBGC formation [17], the formation of FBGCs by adherent macrophages on peptide-grafted networks was modulated by the primary structure of the grafted peptide. Grafted GGGPHSRNGGGGGGRGDG, but not GGGRGDGGGGGGPHSRNG, GGGPHSRNG, nor GGGRGDG, supported higher FBGC density comparable to that of the reference material (11 ± 3 and $23 \pm 9\,\text{cells}\,\text{mm}^{-2}$, $n = 6$, mean $\pm$ SEM, for GGGPHSRNGGGGGGRGDG-grafted surfaces and tissue-culture polystyrene, respectively). These results confirmed the design of bioactive oligopeptides based on the functional architecture of fibronectin. Such peptides were employed to elucidate the molecular mechanism and structural requirement of macrophage adhesion, phenotypic development and to provide methods of manipulating macrophage function for potential cellular/tissue engineering.

The major function of macrophages is to mediate host immune and inflammatory response against foreign objects. Because of this reason, a clear understanding of the complex interaction between macrophage and biomaterial is crucial in the improvement of materials employed in the construction of biomedical devices. Several methodologies were utilized to examine the relationship between material chemistry and macrophage behavior at protein and cellular levels under *in vitro* and *in vivo* conditions. Based on these findings, a series of oligopeptides were designed, synthesized and grafted onto a non-adhesive substrate to study the mechanisms of protein-mediated macrophage function.

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