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The role of complement C3 and fibrinogen in monocyte adhesion to PEO like plasma deposited tetraglyme

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Abstract

The role of complement C3 in mediating adhesion of monocytes to plasma deposited tetraglyme surfaces was studied. Although fibrinogen (Fg) is usually considered the main factor in mediating phagocyte attachment, plasma deposited PEO-like tetraethylene glycol dimethyl ether (tetraglyme) coatings that have ultra-low Fg adsorption ($< 10 \text{ ng/cm}^2$) from low concentration solutions and low monocyte adhesion *in vitro* still show high phagocyte adhesion after short implantations and later become encapsulated when tested *in vivo*. To test whether higher Fg adsorption under *in vivo* conditions could explain the higher *in vivo* reactivity, we again measured the resistance of tetraglyme films to Fg adsorption. We found a surprising and previously unreported increased amount of adsorbed Fg on tetraglyme surfaces from higher concentration protein solutions. However, monocyte adhesion to tetraglyme did not markedly increase despite the increased Fg adsorption. We thus suspected proteins other than Fg must be responsible for the increased *in vivo* reactivity. We found that on tetraglyme pre-adsorbed with C3-depleted serum, monocyte adhesion was greatly reduced as compared to samples adsorbed with normal serum. Addition of exogenous pure C3 to the serum used to pre-adsorb the surfaces restored monocyte adhesion to tetraglyme coatings. While Fg clearly plays an important role in mediating monocyte adhesion to tetraglyme surfaces, the results show an additional role for adsorbed C3 in monocyte adhesion.

Introduction

Monocytes and macrophages are considered to be the key cells involved in the foreign body response (FBR) and so their adhesion to biomaterials has been the subject of many studies [1,2]. Monocyte and macrophage adhesion is mediated by adsorbed proteins that coat the biomaterial almost immediately upon implantation of the device. In various studies, several different proteins have been shown to have the ability to mediate monocyte adhesion, including fibrinogen (Fg) [3], IgG [4], and complement C3 [5]. This is probably because the monocyte $\alpha_M\beta_2$ integrin, also known as CD11b/CD18, CR3, and Mac-1, can bind a variety of ligands, including Fg [6], iC3b, ICAM-1, and coagulation Factor X [7,8]. Nevertheless, much of the recent focus on the interaction between monocytes and macrophages and adsorbed proteins during the initial phases of the FBR has centered on Fg [9].

The major role of adsorbed Fg in monocyte and macrophage adhesion has been shown both *in vitro* and *in vivo* studies. A strong, linear, and positive correlation between the amount of adsorbed Fg (Γ_{Fg}) and *in vitro* monocyte adhesion to a series of chemically modified polystyrene surfaces after 24 hours was observed from both plasma and pure Fg solutions [4]. In another *in vitro* study, a strong positive correlation was observed between the amount of denatured Fg (but not total Fg) on a biomaterial's surface and number of adherent cells [10]. *In vivo* studies of intraperitoneal implants of protein pre-adsorbed poly(ethylene terephthalate) (PET) samples showed a large decrease in phagocyte adhesion when the mice

were depleted of Fg compared to implants in normal mice, a decrease that was overcome if the PET was pre-adsorbed with Fg [3]. In addition, when Fg blood levels were restored in the Fg-depleted mice via Fg injection prior to sample implantation, the number of adherent phagocytes on the implants was as great as on implants in normal mice [3]. The major role of Fg in monocyte and macrophage adhesion shown by these studies prompted us to develop poly(ethylene oxide)-like tetraglyme surfaces that would greatly reduce Fg adsorption as a means to reduce short term phagocyte adhesion and thus reduce the later encapsulation process that is thought to require such adhesion.

Poly(ethylene oxide) (PEO, $\text{HO}-(\text{CH}_2-\text{CH}_2-\text{O})_n-\text{H}$) surface modification and coatings are well known to be effective in reducing non-specific protein adsorption [11,12,13] and subsequent cell adhesion [14,15]. Although the mechanisms by which PEO resists protein adsorption are not fully understood [16,17], it is believed that a major mechanism is the tight binding of water due to strong hydrogen bonding between ether-carbon groups in PEO and water molecules [18]. The bound water is thought to act as a barrier that prevents proteins from adsorbing to a biomaterial's surface. Because of its effectiveness as a non-fouling surface, a variety of methods for generating PEO coatings have been reported, including grafting of PEO chains to surfaces [19], incorporation of PEO to the backbone of polymers to generate co-polymers [20,21,22], generation of hydrogels [23], and generation of self-assembled monolayers [24], among others. However, the ability of these various methods to produce protein and cell repellant surfaces varies widely depending on a number of factors, such as surface coverage, PEO chain density, and PEO chain length. The variable degree of repellency observed is important because, at least with blood platelets, ultralow fibrinogen adsorption must be achieved to prevent platelet adhesion [25].

Our lab has generated radio frequency glow-discharge (RFGD) plasma polymerized PEO-like tetraglyme coatings ($\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_4\text{CH}_3$) displaying ultra-low Γ_{Fg} from low concentration solutions [26]. Tetraglyme coatings display ultralow Fg adsorption ($< 10 \text{ ng/cm}^2$) from 1% plasma or 0.03 mg/ml Fg, and exhibit very low *in vitro* monocyte adhesion [9]. Despite ultralow Γ_{Fg} from low concentration solutions *in vitro*, tetraglyme coated surfaces implanted subcutaneously in rats for one day had a large number of adherent cells, and after 28 days had a thick fibrous capsule formed around the implant [27]. As reported in this paper, we first tried to see if the difference between the *in vivo* and *in vitro* results was due to higher Fg adsorption under *in vivo* conditions, so we evaluated Fg adsorption from higher concentration solutions, 0.3 and 3 mg/ml pure Fg and 10% and 100% plasma, something which had not been previously tested. Unexpectedly, the amount of Fg adsorption to tetraglyme surfaces increased significantly when the surfaces were challenged with the higher concentration solutions. However, the higher Fg adsorption under these conditions only caused a small increase in monocyte adhesion. These findings suggested to us that focusing solely on the role of the single protein Fg in mediating monocyte/macrophage adhesion during the acute phase of the FBR might be inadequate and that instead one or more of the numerous other proteins that are present in the adsorbed layer may also play an important role.

Although complement proteins play a key role in defending the body from anything “non-self”, their role in the foreign body response to biomaterials, and PEO in particular, has been largely overlooked. It has been generally accepted that hydroxyl ($-\text{OH}$) and amine ($-\text{NH}_2$) rich materials promote complement activation, as the presence of these end groups facilitates the formation and covalent binding of the cleaved form of C3 [28]. However, the presence of hydroxyl and amine groups is not necessary for the adsorption of C3 to a biomaterial [28], and recent studies have shown that C3 adsorbs to PEO-thiols on gold surfaces from plasma solutions [24] and to PEG-hydrogels from serum solutions [29]. Furthermore, a number of studies have recently reported the effect of end groups of PEO surfaces on complement

activation [24,30,31,32]. Thus, the studies presented here were therefore primarily designed to elucidate the role of complement C3 in monocyte adhesion to tetraglyme coatings *in vitro*. Radiolabeled Fg and pure C3 were used to measure the amount of adsorption of each protein to tetraglyme coated and the uncoated control surfaces. In addition, primary human monocyte adhesion to tetraglyme coatings pre-adsorbed with selectively depleted sera or plasma was evaluated. Protein solutions tested include normal human serum and plasma, complement C3 depleted serum, Fg-deficient plasma, as well as C3 and Fg repleted serum and plasma, to better understand the mechanisms at play in the body using a simplified *in vitro* model. The results show that complement C3 plays an important role in mediating monocyte adhesion to tetraglyme surfaces in addition to Fg. Related studies, including evaluations of complement activation by tetraglyme, are presented elsewhere [33].

Materials and Methods

Fluoroethylene propylene (FEP, $(\text{CF}(\text{CF}_3)\text{-CF}_2(\text{CF}_2\text{-CF}_2)_n)_m$) film was a gift from Du Pont de Nemours & Co. (Circleville, OH). The film was received in 0.125 mm thick sheets, and was cut into 7.9 mm diameter disks using a die punch. Once punched, samples were cleaned by two successive ultrasonicated treatments in methylene chloride, acetone, and methanol, for 10 minutes each. FEP disks were left to dry in a laminar flow hood, and stored at room temperature in a clean polystyrene Petri dish with its polystyrene lid held in place with parafilm, until ready to use. Tetraethylene glycol dimethyl ether (tetraglyme, $\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_4\text{CH}_3$) monomer was purchased from Sigma-Aldrich (St. Louis, MO).

Buffers and reagents

Citrate phosphate buffered saline (CPBS) contained 10 mM Na_2HPO_4 , 10 mM citric acid, and 120 mM NaCl, pH 7.4. Citrate phosphate buffered saline containing 3 mM NaN_3 and 10 mM NaI (CPBSzI) (pH 7.4) was used for protein adsorption. Dulbecco's phosphate buffered saline (DPBS), Histopaque® Triton-X 100, and trypan blue stain were purchased from Sigma-Aldrich (St. Louis, MO). Heparinized and plain vacutainers were purchased from Becton Dickinson (Franklin Lakes, NJ). Purified human fibrinogen (> 95% clottable) was purchased from Enzyme Research Laboratories (South Bend, IN). Purified whole complement C3 ($\geq 95\%$ pure) was purchased from Sigma-Aldrich (St. Louis, MO), EMD Biosciences (San Diego, CA), and also from RDI Division of Fitzgerald Industries International (Concord MA). By "whole", we mean the full sized C3 molecule as opposed to any of its fragments such as C3a. Complement C3-depleted serum was purchased from EMD Biosciences (San Diego, CA). Fibrinogen-deficient plasma was purchased from George King Biomedical, Inc. (Overland Park, KS). RPMI-1640 with L-Glutamine and ethylenediaminetetraacetic acid (EDTA) was purchased from Invitrogen (Carlsbad, CA). Monocyte cell culture media for monocyte adhesion studies consisted of RPMI-1640 (Invitrogen) supplemented with 25 mM HEPES (Gibco BRL), 1 mM MEM sodium pyruvate (Sigma), 0.1 mM MEM non-essential amino acids (Sigma), and 1% penicillin-streptomycin (Sigma).

Radio-frequency glow discharge plasma coatings

A previously described capacitively coupled radio-frequency glow discharge plasma system [26] was used to generate our tetraglyme coatings. Briefly, the system consists of a vacuum pump, a mass flow controller to determine the flow rate of monomer and gas, a monomer reservoir, copper electrodes, a cylindrical reaction chamber for sample preparation, and a 13.56 MHz radio-frequency power source.

FEP substrates were hung from fishing line strung between the posts of a glass frame, to ensure that both sides of the disks were coated at once. This set-up eliminated the need for

two runs to coat both sides of the substrate and the resulting damage on the side coated first, which has been observed previously by the first author (unpublished observations) and others in our group [34]. After FEP substrates were loaded at a position 1 to 3 inches upstream of the RF coil of the plasma reactor, they were treated with a 3-minute argon plasma generated using 40W and 350 mtorr to etch away surface impurities and introduce free radicals to promote covalent attachment of the tetraglyme coating. The Ar was completely evacuated from the reactor chamber, and tetraglyme monomer vapor was introduced. Tetraglyme was then polymerized by subjecting it to an initial 80W 1-minute step immediately followed by a 20-minute 10W step at 350 mtorr. Samples were then stored in the same way as the cleaned, uncoated samples until ready for use.

Fibrinogen radiolabeling

We used the ICI method, developed by Helmkamp *et al* [35] as modified by Horbett [36], to radiolabel human fibrinogen (Fg) with Na¹²⁵I (Perkin Elmer, Waltham, MA), using a 2:1 ICI to Fg molar ratio. Unbound ¹²⁵I was separated from the labeled protein by two passes through desalting chromatography columns (Econo-Pac 10DG, BioRad, Hercules, CA). Radiolabeled Fg was stored at -80°C, and used within four weeks. Prior to Fg adsorption studies, enough ¹²⁵I Fg was added to pure Fg solutions (0.03, 0.3, and 3 mg/ml Fg) and pooled plasma solutions (number of donors = 3) (1%, 10%, and 100%) to achieve a specific activity no less than 40 cpm/ng. Because the specific activity of the stock ¹²⁵I Fg was high, the amount of ¹²⁵I Fg added to the various protein solutions was small and did not significantly change the protein concentration.

Complement C3 radiolabeling

Shortly before use, two Iodobeads (Pierce Biotechnology, Rockford, IL) were washed with 1 mL PBS, and dried on filter paper to remove any loosely bound particles. Iodobeads were added to 0.25 mL PBS, and 1mCi Na¹²⁵I (Amersham) was added to the beads and allowed to react for 5 minutes at room temperature. Next, 0.5 ml of a 0.2 mg/ml pure C3 solution in PBS was added to the Iodobeads, and allowed to react for 15 minutes at room temperature. Unbound ¹²⁵I was removed and stored as done for ¹²⁵I-Fg. ¹²⁵I incorporation rates of approximately 40% were achieved. Prior to C3 adsorption studies, enough ¹²⁵I C3 was added to 1% or 10% heparinized plasma or serum solutions to achieve a specific activity no less than 40cpm/ng. The specific activities of the protein solutions were determined by collecting a small aliquot of each solution and measuring the bound ¹²⁵I using a Cobra II gamma counter. Because the specific activity of the ¹²⁵I C3 was high, the amount of ¹²⁵I C3 added to the different protein solution was small and did not significantly change the protein solution concentration.

Protein adsorption

FEP and tetraglyme coated FEP samples were equilibrated by submersion in degassed CPBSzI for 1 hour at 37°C. Next, the soaking buffer was replaced with the protein solution of choice at a specific activity of 40 cpm/ng and the samples incubated for two hours at 37°C. The samples were rinsed free of bulk protein with CPBSzI by sequentially dip rinsing in fresh buffer three times. The bound ¹²⁵I was measured using the Cobra II gamma counter. Adsorption of Fg (Γ_{Fg}) and pure C3 (Γ_{C3}) (ng/cm²) was calculated from the bound ¹²⁵I using the specific activities of the protein and the surface area of both sides of the sample, with corrections for background radioactivity and decay.

Monocyte isolation

Monocytes were isolated by magnetic activated cell sorting (MACS®, Miltenyi Biotec, Auburn, CA) from healthy human blood collected from venipuncture by a trained

phlebotomist at the University of Washington Medical Center, under a protocol approved by the UW Human Subjects Committee. The following protocol for the depletion of all non-monocyte cells was adapted from the MACS® negative selection protocol provided by Miltenyi Biotec. Heparinized blood (143 USP units of sodium heparin, BD Biosciences), diluted 1:1 in sterile DPBS containing 2 mM ethylenediaminetetraacetic acid (EDTA, Invitrogen, Carlsbad, CA) was carefully layered over 15 ml Histopaque® 1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 400 g for 30 minutes at 25°C. The leukocyte-rich buffy coat at the interface was collected, and washed twice in DPBS. The washed cells were resuspended with 10 ml MACS® buffer, containing DPBS plus 2 mM EDTA and 0.5% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), and counted using a hemocytometer. Non-monocyte cells in the fraction were depleted using the biotin-conjugated antibody cocktail against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a antibodies, following the manufacturer's instructions. Isolated cells were more than 95% viable, as determined by Trypan blue exclusion, and monocytes typically made up more than 90% of the cells collected, as determined by light microscopy.

Monocyte adhesion

Prior to addition of monocytes, the tetraglyme or FEP disks were pre-equilibrated in buffer as in the adsorption studies, then pre-adsorbed by exposure to protein solutions, and finally rinsed with buffer. Rinsing was done with DPBS by removing the protein solution and replacing it with buffer with a pipette. The buffer was then removed and replaced with fresh buffer two more times. Samples were pre-adsorbed with 10% serum, 10% plasma, 10% C3-depleted serum, or 10% C3-depleted serum plus 125 µg/µl pure C3 for two hours at 37°C, or sequentially pre-adsorbed with 0.3 mg/ml Fg and 10% serum or 10% C3-depleted serum. After the pre-adsorption and rinsing steps, the samples were transferred to a sterile 48-well tissue culture plate. The pre-adsorption solutions used were collected and saved to supplement the media used for the cell adhesion study.

Isolated monocytes were suspended in RPMI-1640 (Invitrogen) supplemented with 25 mM HEPES (Gibco BRL), 1 mM MEM sodium pyruvate (Sigma), 0.1 mM MEM non-essential amino acids (Sigma), and 1% penicillin-streptomycin (Sigma) at a concentration of 1×10^6 cells per ml. The cells were allowed to rest in a polypropylene conical tube at 37°C and 5% CO₂ for one hour after the isolation procedure. Next, the cells were re-suspended by mixing, and 200 µl of the cell suspension supplemented with 200 µl of the desired protein solution reserved from the protein pre-adsorption step was added to each of the pre-adsorbed samples. Monocytes were allowed to adhere for 2 hours at 37°C and 5% CO₂. After adhesion, samples were rinsed by pipetting and removing 400 µl fresh RPMI-1640 at 37°C into each well three times. This critical step was implemented in order to remove any non-adherent cells from the biomaterials' surface without disturbing adherent monocytes.

The number of adherent monocytes was quantified using a modified lactate dehydrogenase (LDH) method [25]. Briefly, samples were moved to a fresh 48-well plate, and cells were lysed by adding 200 µl of a 1% Triton-X solution (v/v) for 30 minutes at room temperature, while shaking on an orbital shaker at 100 rpm. Next, the cell lysate was mixed with 200 µl of the LDH substrate (cytotoxicity kit, Roche Applied Science, Indianapolis, IN), and allowed to incubate for 15 minutes at 37°C. 100 µl of 1N HCl was added to stop the reaction. Then, 250 µl of the solution was transferred from each well to a fresh 96-well plate, and the absorbance was read at 490 nm and at the reference wavelength of 650 nm ($A_{490-650}$ value) using a microplate reader (Molecular Devices Corporation, Union City, CA). A calibration curve relating number of cells to absorbance measurements was generated by lysing known numbers of cells, and conducting the LDH assay as described above. The calibration curves were highly linear ($R^2 > 0.95$) and reproducible in the range of 0 to 2×10^5 cells.

Donor variation can significantly affect monocyte behavior, and different donors were used for each monocyte isolation. Thus, to reduce the effect of donor variation, adhesion to samples of the various materials were always measured with the monocytes from a single donor. However, the studies presented here were duplicated two separate times, using a different donor for each monocyte isolation. In addition, the normal human serum and plasma used in these studies was pooled from 3 different donors from each study. Similar trends were observed each time..

Statistical analysis

To determine the statistical significance of the data, a Student's paired t-test was performed using EXCEL (Microsoft Corporation, Seattle, WA). The level of significance was $p < 0.05$.

Results

Fibrinogen adsorption studies

Γ_{Fg} to tetraglyme coated FEP and uncoated FEP films was measured from a series of pure Fg solutions (Figure 1.a) as well as a series of normal human plasma solutions (Figure 1.b) using radiolabeled Fg. Γ_{Fg} from pure Fg solutions ranging in concentration from 0.03 to 3 mg/ml Fg to the tetraglyme coated FEP was much lower than that observed on uncoated FEP films (83% reduction on average). The ultralow protein adsorption of tetraglyme coatings previously reported[9] was observed when samples were pre-adsorbed from 0.03 mg/ml Fg solutions, with approximately 6 ng/cm² Fg being adsorbed onto tetraglyme coated FEP. However, upon increasing the Fg concentration we observed significantly higher adsorption, as Γ_{Fg} on tetraglyme coatings increased to approximately 65 and 83 ng/cm² at 0.3 and 3 mg/ml Fg respectively, much higher than the 6 ng/cm² we consider to be in the "ultralow" range. Γ_{Fg} on FEP from each of these solutions was 52, 222, and 762 ng/cm², respectively.

Γ_{Fg} from plasma solutions, ranging in concentration from 1% to 100% normal human plasma, was also tested (Figure 1.b). Γ_{Fg} to samples equilibrated to plasma increased with plasma concentration, similar to what we observed as with pure Fg solutions, but the overall amounts adsorbed were lower, due to increased competition from other proteins for adsorption on the surface. As was the case with pure Fg solutions, Γ_{Fg} to tetraglyme coatings was what we have called "ultralow" for samples exposed to low concentration plasma (4 ng/cm² from 1% plasma solution), as previously observed. But the Γ_{Fg} on tetraglyme coated FEP increased with increasing plasma concentration, reaching 18 and 55 ng/cm² from 10% and 100% plasma solutions, respectively. Γ_{Fg} to FEP from plasma was again significantly higher than that to tetraglyme coatings, adsorbing approximately 44, 51, and 123 ng/cm² from 1, 10, and 100% plasma solutions, respectively. These experiments were reproduced with three different batches of tetraglyme coated FEP and pooled human plasma, and similar results were obtained each time.

Effect of increased surface fibrinogen on monocyte adhesion

Monocyte adhesion to tetraglyme coated FEP pre-adsorbed with pure Fg solutions was significantly lower than that observed on FEP samples for all Fg concentrations tested (Figure 2.a). Adhesion to tetraglyme coatings increased with increasing Γ_{Fg} for samples pre-adsorbed with concentrations between 0.03 and 0.3 mg/ml Fg. However, the number of adherent monocytes on surfaces exposed to higher Fg concentrations did not continue to increase despite increased Γ_{Fg} on tetraglyme coatings pre-adsorbed with up to 3 mg/ml Fg. Similarly, adhesion on FEP samples pre-adsorbed with increasing amounts of adsorbed Fg increased for samples adsorbed with concentrations from 0.03 to 0.3 mg/ml Fg, but adhesion to FEP samples pre-adsorbed with 3 mg/ml Fg showed no further increase.

Monocyte adhesion to tetraglyme coated and uncoated FEP samples pre-adsorbed with 1 to 100% plasma is shown in Figure 2.b. Monocyte adhesion to these samples greatly differed from that observed on samples pre-adsorbed with pure Fg solutions. A marked increase in the number of adherent monocytes to tetraglyme coatings pre-adsorbed with increasing plasma solution concentration was observed. Conversely, monocyte adhesion to FEP pre-adsorbed with increasing plasma solution concentrations actually decreased. To our surprise, the number of adherent monocytes to tetraglyme coatings pre-adsorbed with 100% plasma was slightly higher than the number of adherent monocytes to FEP pre-adsorbed with the same concentration solution. The adhesion data for samples pre-adsorbed with plasma solutions differed greatly from that observed for samples pre-adsorbed with pure Fg containing equivalent concentrations of Fg, suggesting that the trends observed on samples treated with plasma solutions do not stem from the involvement of Fg alone. Thus, we became interested in the role of complement proteins in mediating monocyte adhesion.

The results reported here were reproduced two separate times using different batches of tetraglyme films and different lots of pooled normal human plasma (number of donors for each lot of pooled plasma = 3).

Complement C3 adsorption studies

Complement C3 adsorption on tetraglyme coated FEP and uncoated FEP surfaces was measured after exposure to 1% or 10% normal human serum or 1% or 10% normal human plasma, using ^{125}I labeled C3 (Figure 3). Γ_{C3} on tetraglyme coatings was lower than that to FEP, though only the difference observed from 1% plasma was deemed statistically significant. Γ_{C3} was highest on both materials from 1% serum, with adsorption to 10W tetraglyme decreasing in the order of 1% serum > 10% serum > 10% plasma > 1% plasma. In the case of FEP, Γ_{C3} decreased in the order of 1% serum > 10% serum > 1% plasma > 10% plasma. The maximum amount of Γ_{C3} on tetraglyme coatings and FEP films, observed from 1% serum, was 11 ng/cm² and 37 ng/cm² C3, respectively.

Effect of complement C3 on monocyte adhesion

As shown in Figure 4, pre-adsorption with complement C3 depleted serum resulted in a large reduction in the number of adherent monocytes on both tetraglyme coated FEP and FEP. Pre-adsorption with C3 depleted serum replenished with pure C3 partially restored monocyte adhesion to tetraglyme surfaces, but did not have a major effect on FEP surfaces. The addition of 0.3 mg/ml Fg to C3-depleted serum restored monocyte adhesion to the levels seen with 10% plasma on tetraglyme coated surfaces. The addition of Fg to C3-depleted serum resulted in a greater number of adherent monocytes to FEP than normal plasma or 10% normal serum with 0.3 mg/ml Fg, though the difference was not statistically significant.

Monocyte adhesion to tetraglyme surfaces pre-adsorbed with 10% normal human serum was a little higher (by about 10–15%) than that observed to 10% normal human plasma adsorbed tetraglymes (Figure 4), whereas the opposite trend was observed for FEP. The addition of 0.3 mg/ml Fg to 10% serum resulted in a sharp increase in monocyte adhesion in both tetraglyme and FEP as compared to 10% serum. The results reported here were reproduced two separate times using different batches of tetraglyme films and different lots of C3-depleted serum, pooled normal human serum (number of donors per lot of pooled serum = 3), and pooled normal human plasma (number of donors per lot of pooled plasma = 3).

Discussion

Fg Adsorption and its effect on monocyte adhesion

The importance of Fg in mediating monocyte adhesion to biomaterials has been carefully studied and seems well established [3,10], and the specific regions on the Fg molecule involved in monocyte adhesion have also been identified [6,37]. However, despite the low fibrinogen adsorption and monocyte adhesion we observed for tetraglyme in our *in vitro* studies, they exhibited relatively high phagocyte attachment after one day *in vivo* and were encapsulated at longer implant times [27]. This led us to reexamine how well our previous *in vitro* studies mimicked *in vivo* conditions, and in particular whether we had used adsorption conditions which were stringent enough. We thought that perhaps the tetraglyme samples became more fouled with Fg than we had previously reported.

We therefore chose to test protein adsorption to tetraglyme and uncoated FEP films from a wider variety of pure Fg solutions (0.03–3 mg/ml, Figure 1.a) and plasma solutions (1% to 100% plasma, Figure 1.b) than we had done in prior studies. We again found that tetraglyme samples adsorbed much less Fg than FEP under all conditions tested, and that Γ_{Fg} under lower protein concentrations (0.03 mg/ml Fg and 1% plasma) was “ultralow” ($<10 \text{ ng/cm}^2$) as previously reported [9]. However, when higher concentration solutions were used, we did find a significant increase in Γ_{Fg} on tetraglyme coatings, reaching a maximum of 83 ng/cm^2 from a 3 mg/ml pure Fg solution. Because these results were unexpected, we did additional controls to exclude the effects of free iodine adsorption or preferential adsorption experiments (not shown) [38]. The reason for this increased protein adsorption remains unclear, although they clearly reflect the existence of low affinity interactions that are not evident until the tetraglyme samples are exposed to much higher protein concentrations than previously tested. Nevertheless, these results indicate that there is an unexpected amount of adsorbed Fg on the tetraglyme surfaces, which could impact the *in vivo* behavior of our surfaces. These new results thus highlight an important extension of our previous understanding of how nonfouling the tetraglyme surfaces really are under conditions closer to those that exist *in vivo* with regard to Fg concentrations.

However, the increased Γ_{Fg} at higher concentrations of Fg in buffer was not accompanied by a corresponding increase in monocyte adhesion. Instead, only small increases in the number of adherent monocytes to the tetraglyme samples pre-adsorbed at higher Fg concentrations were observed (Figure 2.a). Even after exposure to higher Fg concentrations and the consequent increase in Γ_{Fg} , monocyte attachment to the tetraglymes is still much lower than to FEP, so tetraglymes still exhibit good cell repellency under these more stringent conditions.

The lack of a proportional increase in the number of adherent cells on tetraglyme samples to the increased amount of adsorbed Fg was most clearly evident on samples pre-adsorbed with 3 mg/ml Fg. For these samples, there was no further increase in adhesion, despite adsorbing the highest amount of Fg of all the tetraglyme samples tested. Similar lack of proportionality was observed for the FEP samples: adsorbed Fg on these surfaces increased from 52, 222, to 762 ng/cm^2 Fg from each of the solutions tested, but a corresponding increase in the number of adherent monocytes in the same magnitude was not observed. The lack of proportional increases in adhesion to the increased Fg adsorption are similar to findings by Tang et al, who showed that phagocyte adhesion was better correlated with denatured fibrinogen than with total fibrinogen [10]. In a forthcoming publication [38], we used antibody binding to show that the availability of the cell binding domain in Fg adsorbed to tetraglymes was well correlated with monocyte adhesion, unlike total Fg adsorption, presumably as a result of conformational changes that expose binding sites.

As a still more stringent test of cell repellency, we tested the ability of tetraglyme coatings to resist monocyte adhesion after exposure to blood plasma, a more physiologically relevant protein solution. Surprisingly, monocyte adhesion to tetraglyme surfaces pre-adsorbed with increasing concentration plasma solutions displayed a marked increase, quite unlike the slight changes observed when tetraglyme was pre-adsorbed with pure Fg. These results were doubly surprising to us because the total amount of Fg adsorbed to tetraglymes from plasma was much lower than from pure Fg solutions. These findings strongly suggested the important of another plasma protein besides Fg.

Complement C3 adsorption and its effect on monocyte adhesion

We thought that the most likely protein besides Fg to be important in monocyte adhesion was C3, as we now explain. First, C3 has been previously shown to participate in monocyte adhesion to some surfaces [5]. Secondly, recent studies in our lab showed that tetraglyme coatings were complement activating [33]. Thus, we measured Γ_{C3} to tetraglyme coatings and FEP films exposed to 1% or 10% normal human serum or heparinized plasma using radiolabeled C3. We found that Γ_{C3} from 1% or 10% normal human serum and plasma to tetraglyme was low, in amounts similar to Γ_{Fg} to tetraglyme films from Fg concentrations equal to the C3 concentration used. Γ_{C3} on FEP was higher from all solutions studied than to tetraglyme coated FEP. Γ_{C3} was highest on both surfaces from 1% serum, probably because of reduced competition for adsorption at the lower serum concentration and the absence of Fg. Similarly, the lower Γ_{C3} from 1% plasma is probably due to increased competition in the presence of Fg.

Although Γ_{C3} was low, even small amounts of adsorbed proteins are known to mediate cell responses. For instance, Tsai et al [25] found that when the amount of adsorbed Fg was above about 10 ng/cm², platelet adhesion reached maximal levels, and prevention of platelet adhesion required that the amount of adsorbed Fg be reduced to “ultralow” levels, i.e. below 5 ng/cm². Therefore, we investigated the role of C3, as compared to Fg, in monocyte adhesion by pre-adsorbing tetraglyme coated FEP and FEP surfaces with a variety of protein solutions, including normal human plasma and serum (pooled, number of donors = 3), as well as C3-depleted serum and Fg-deficient plasma (Figure 4). Monocyte adhesion to tetraglyme or FEP films was greatly reduced when 10% C3-depleted serum was used to pre-adsorb the surfaces. When C3 was restored to normal concentrations by adding 125 µg/ml purified C3 to 10% C3-depleted serum and the C3 replenished serum was used for pre-adsorption, monocyte adhesion to tetraglyme was almost the same as after pre-adsorption with normal serum.

The reduction of adhesion to surfaces pre-adsorbed with C3 depleted serum and the restoration of adhesion after C3 replenishment clearly show that the presence of C3 is important in monocyte adhesion to tetraglyme coatings. On the other hand, monocyte adhesion to pre-adsorbed FEP was not restored to normal levels when purified C3 was added to C3-depleted serum used for pre-adsorption, despite the relatively high levels of adherent monocytes on FEP surfaces treated with normal human serum. These differences are possibly related to the fact that tetraglyme is a complement activating material, whereas FEP is not [33].

The addition of Fg to normal human serum used for pre-adsorption resulted in the highest number of adherent monocytes to tetraglyme coatings of any condition tested, suggesting there might be a synergistic effect between C3 and Fg. The need for both proteins to support maximal adhesion is also supported by the fact that adhesion to tetraglymes pre-adsorbed with C3 deficient serum to which only Fg had been added was not as high as when both proteins were present. Fg is known to mediate monocyte adhesion via interaction with the $\alpha_M\beta_2$ integrin. Thus, we expected monocyte adhesion to be restored to some degree when Fg

was added to C3-depleted serum. However, while the mechanism of interaction between Fg and monocytes is known to involve an adhesion receptor, the mechanism involved in mediating monocyte adhesion by tetraglyme-adsorbed C3 may be more complex. This is because we have shown in a forthcoming publication[33] that tetraglyme coatings are complement activating, so the enhanced adhesion in the presence of C3 could be due to C3a activation of monocytes as well as cell adhesion to tetraglyme via interactions with cell surface receptors. Despite this uncertainty as to the mechanism, the importance of C3 in monocyte adhesion is strongly supported by the experiments presented here.

All of the monocyte adhesion studies presented here were conducted using cell culture media supplemented with 200 μ l of the protein solution used to pre-adsorb that sample. This was done in order to closer mimic *in vivo* settings using our *in vitro* model. Rinsing the samples with buffer after the pre-adsorption step artificially removes bulk and loosely bound proteins, which may otherwise be involved in monocyte adhesion *in vivo*. By replenishing the cell culture media with small amounts of the protein solution used to pre-adsorb the samples, we are maintaining a protein-rich environment without introducing any additional proteins via the addition of serum to the media.

This work focused on what we consider to be a key and critical step in the foreign body response: monocyte adhesion as a result of protein adsorption on a biomaterial's surface. The behavior of adherent monocytes beyond the two-hour time point was not studied, so our results are probably most relevant to the initial cell-protein interactions that might take place soon after a biomaterial is implanted *in vivo*. While other approaches, including cytokine release evaluation and cytoskeleton structure changes, certainly provide valuable information regarding the activation of adherent monocytes, it is important to stress that those events would not occur without initial monocyte adhesion to surfaces. This critical role of the adhesion step led us to design these experiments as presented here. Nevertheless, other types of information, such as the morphology of the cells on the surfaces and inflammatory protein release by monocytes beyond the two-hour time point would provide further understanding of the effect of adsorbed complement C3 and are thus appropriate for future studies. In fact, recent studies using LC-MALDI to study the global proteome of adherent monocytes highlight the importance of gaining a better understanding of protein expression as a result of protein-mediated cell-biomaterial interactions [40].

The studies presented here were duplicated two separate times, using different donors for monocyte isolation and serum and plasma generation, and similar trends were observed each time, i.e. monocyte adhesion was greatly decreased in the absence of C3 and restored by addition of pure C3. Thus, the results support a causal relationship between whole C3 adsorption and monocyte adhesion. Further work, such as a study of the time course of the effect of protein adsorption on monocyte adhesion would probably strengthen our basic findings, e.g. to show over what period of time the monocyte adhesion is decreased in the absence of C3 and are recommended for future studies.

Finally, though we have designed our studies to evaluate the importance of C3 and Fg in monocyte adhesion by carefully choosing our protein solutions, our studies do not rule out an additional role for other proteins present in human serum and plasma. The use of serum and plasma solutions added a complexity to our design in that many other proteins besides those we studied were present. Some of these proteins have been shown to impact the foreign body response to biomaterials. For instance, fibronectin has been shown to play an important role in the chronic fibrotic stage of the foreign body response, though does not affect leukocyte recruitment [39]. Certainly, a better understanding of the role of the other adhesive proteins present in normal blood serum and plasma in monocyte adhesion to tetraglymes and other biomaterials would be helpful to the engineering of better

biomaterials. However, the role of fibronectin and other proteins must await future studies, as we chose to focus here on the role of complement protein C3 and on the role of increased amounts of adsorbed Fg in monocyte adhesion to tetraglyme. The studies we report here, using selectively depleted serum and plasma and performing replenishment studies of the depleted proteins to create conditions that more closely simulate physiologically relevant conditions are significant because they clearly show the importance of both C3 and Fg in mediating monocyte adhesion to tetraglyme surfaces.

Conclusion

The importance of C3 in addition to Fg in mediating monocyte adhesion is well supported by the findings in this study. Although we found C3 adsorbed only in small amounts, the importance of this protein in monocyte adhesion was confirmed, as monocyte adhesion to tetraglyme was impaired when C3 deficient serum, rather than normal human serum, was used to pre-adsorb the surfaces. Cell adhesion was restored upon replenishing the C3-depleted serum solution with purified C3. The presence of Fg in the pre-adsorption solutions further increased monocyte adhesion, indicating two separate mechanisms for the adhesion of inflammatory cells on our surfaces. The finding that C3 is also involved in monocyte adhesion to tetraglyme coatings helps explain the poor correlation with Fg adsorption in our *in vitro* studies. These results also suggest that the ability of these materials to support monocyte adhesion and encapsulation *in vivo* despite the low Fg adsorption might be due to C3 adsorption, but further investigation would be needed to test that idea. Another important conclusion from our studies is that the ultralow fouling characteristic of tetraglyme occurs only when tested with low concentration protein solutions, with greater amounts of Fg adsorbing when high concentration solutions were used, which may also help explain the *in vivo* phagocyte adhesion and encapsulation.

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Figure 1.

Fg adsorption to tetraglyme and FEP materials from a) 0.03, 0.3, and 3 mg/ml pure Fg solutions and from b) 1%, 10%, and 100% plasma solutions. Data are displayed as mean $\pm$ SEM; n = 3. Asterisks denote statistically significant differences in the amount of adsorbed Fg onto tetraglyme as compared to FEP ($p = 0.05$).

Figure 2.

Two hour monocyte adhesion to tetraglyme and FEP surfaces pre-adsorbed with a) purified Fg solutions and b) normal human plasma. Data are displayed as mean $\pm$ SEM; $n = 4$. Asterisks denote statistically significant differences in the number of adherent monocytes on tetraglyme as compared to FEP for a given protein concentration ($p = 0.05$).

Figure 3.

Complement C3 adsorption to tetraglyme and FEP materials from 1% or 10% normal human serum or 1% or 10% normal human plasma. Data are displayed as mean $\pm$ SEM; $n = 3$. The single asterisk denotes the statistically significant difference in amount of adsorbed C3 from 1% plasma as compared to FEP ($p = 0.05$). The # denotes the statistically significant difference in the amount of adsorbed C3 from 10% serum and 10% plasma as compared to FEP ($p = 0.10$).

Figure 4.

Two hour monocyte adhesion to protein pre-adsorbed tetraglyme and FEP surfaces. Data are displayed as mean $\pm$ SEM; $n = 4$. Asterisks denote statistical significant differences in numbers of adherent monocytes in the pairs connected by arrows ($p = 0.05$).