Cell and protein adhesion studies in glaucoma drainage device development

The AGFID project team*

Abstract

***Aim*—To examine in vitro whether phosphorylcholine coating of poly(methylmethacrylate) can reduce the adhesion of fibrinogen, fibrin, human scleral fibroblast and macrophage compared with current biomaterials used in the construction of glaucoma drainage devices.**

***Methods*—Sample discs (n=6) of poly(methylmethacrylate), silicone, polypropylene, PTFE, and phosphorylcholine coated poly(methylmethacrylate) were seeded with fibrinogen, fibrin, fibroblast, and macrophages and incubated for variable lengths of time. The quantification was performed using radioactivity, spectrophotometry, ATP dependent luminometry, and immunohistochemistry respectively.**

***Results*—Fibrinogen and fibrin adhesion to phosphorylcholine coated poly(methylmethacrylate) were significantly lower than PMMA (p=0.004). Phosphorylcholine coating of poly(methylmethacrylate) also significantly reduced the adhesion of human scleral fibroblast (p=0.002) and macrophage (p=0.01) compared with PMMA. All the other biomaterials showed either similar or insignificantly different levels of adhesion to all the proteins and cells tested compared with PMMA.**

***Conclusion*—Phosphorylcholine coating is a new material technology that offers considerable promise in the field of glaucoma drainage device development.**

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Valve mechanisms in glaucoma drainage devices (GDDs) have largely failed to provide consistent flow control and protection from hypotony. Limitations of currently marketed valved devices have recently been exposed in theoretical,[1] experimental,[2 3] and clinical examinations.[4]

Flow control in non-valved devices is critically dependent on the internal dimensions of the flow passage. For tube devices, resistance varies with the fourth power of tube diameter in accordance with Poiseuille's law.[5] Small changes in diameter induced by protein or cellular spoilation may lead to substantial changes in flow performance. Resistance to surface spoilation is therefore a key requirement for materials used in GDDs which seek to control flow using single or multiple small bore tubes as flow resistors.

A new class of polymers synthesised from monomers based on phosphorylcholine (PC) offers a promising approach to improving the biocompatibility of medical devices. PC is the hydrophilic head group of the predominant phospholipids (lecithin and sphingomyelin) in the outer envelope of mammalian cell membranes.[6–8] As coatings or bulk materials, these "biomimetic" polymers are thought to have enhanced resistance to non-specific protein adhesion. Early applications of this technology have included chest drains[9] and contact lenses.[10] We are currently examining the possible role of this class of biomimetic polymers as suitable candidate materials for GDD construction.

Protein and cellular components, which are likely to play an important part in tube surface spoilation in the postoperative aqueous environment, include fibrinogen, fibrin, and macrophages. Small bore filtration channels may also be capped off externally by scleral fibroblasts growing into a fibrin scaffold.[11] This study set out to examine the adhesion of each of these elements in vitro, comparing PC coated poly(methylmethacrylate) (PMMA) and materials used in existing GDDs (Table 1).

Methods

MATERIALS PREPARATION

Sample discs (8 mm diameter) were punched from 0.5 mm thick sheet PMMA cast from polymer beads (ICI, London), polypropylene (Goodfellow, Cambridge), PTFE (Goodfellow, Cambridge), and silicone (Goodfellow, Cambridge). Additional PMMA discs were coated with PC1008 (2:1 copolymer of lauryl methacrylate and methacryloyl phosphorylcholine) (Biocompatibles, Farnham). Sample discs were washed sequentially with a 7X-PF detergent (ICN Pharmaceuticals, Hants) and distilled water. The coated discs were then air dried in sterile conditions for 10–15 minutes at room temperature before experimentation. Six discs of each material were used for each of the adhesion assays.

ADSORPTION OF FIBRINOGEN

I^{125} labelled fibrinogen (ICN Pharmaceutical, Hants) was diluted (ratio 1:9) with unlabelled human fibrinogen (Sigma, Dorset) in 12.6 ml

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Table 1 Materials used in contemporary glaucoma drainage devices (GDDs)[4]

GDDs	*Year of introduction*	*Tube material*	*Plate material*
Molteno	1979	Silicone	Polypropylene
Baerveldt	1990	Silicone	Silicone
Krupin with disc	1990	Silicone	Silicone
Ahmed	1993	Silicone	Polypropylene with silicone valve
Optimed Model-1014	1995	Silicone	Silicone with PMMA matrix
Helies	1997	N/A	PTFE

of phosphate buffered saline (PBS). A total of 25.2 ng of fibrinogen in 40 µl of solution was pipetted onto each disc in a 24 well plate and incubated at room temperature for 2 hours. The quantity was chosen to enable mid-range readings in the scintillation counter. The discs were then washed (×3) in PBS, transferred to scintillation vials and submerged in scintillation fluid. The scintillation count for each vial was then measured over 1 minute in a liquid scintillation counter (Model 1209 Rack Beta, LKB Ltd, USA).

ADHESION OF FIBRIN

Fresh venous blood (20 ml) was obtained from a healthy adult male volunteer in a citrated vial to prevent clotting. The blood was centrifuged for 10 minutes at 3000 rpm to obtain plasma. Plasma 0.1 ml was diluted with 1 ml of normal saline; 0.1 ml of 0.025M calcium chloride solution was then added to this mixture to initiate clotting; 0.2 ml of this solution, which covered the entire surface of the disc, was immediately pipetted onto centre of each sample disc. These discs were incubated for 2 hours at room temperature to allow the formation and adherence of fibrin, before being washed (×3) in PBS, and transferred to a sterile 24 well plate. Fibrin deposits were dissolved by adding 0.2 ml of 4M guanidine thiocyanate into each well with constant agitation (30 rpm) for 1 hour at room temperature. The tyrosine residue in 0.1 ml of this solution was measured as a quantification marker for dissolved fibrin using a spectrophotofluorometer (Model 7687, American Instrument Company, MD, USA) (excitation wavelength 275 nm; emission wavelength 310 nm).

ADHESION OF SCLERAL FIBROBLASTS

Eye bank scleral rim tissue from adult donors (Moorfields Eye Hospital Eye Bank) was cultured to produce a strain of scleral fibroblasts in Eagle's minimal essential medium (MEM) (Gibco, Life Technology Ltd, Paisley) supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. Cells were serially passaged to 6–7 population doubling by standard trypsin-EDTA dispersion. The fibroblasts were then seeded in MEM at a density of 6000 cells/cm^2 into wells containing the sample discs, and incubated at 37°C under 5% carbon dioxide to allow attachment for 48 hours. This cell density means that the total number of cells delivered was higher than required to cover the entire surface of the disc. The discs were then washed (×3) in PBS and transferred to a new 24 well plate. Intracellular ATP was liberated by incubating each disc with 100 ml of sterile hypotonic lysis buffer (0.01M TRIS-acetate pH 8, 2 mM EDTA). After 30 minutes the ATP solution was diluted 1:1 with an additional buffer (0.2M TRIS-HCl, 0.2 mM EDTA, pH 7.3). ATP levels in this buffered lysate were then measured as a quantification marker for the adherent fibroblasts by ATP dependent luminometry, using a commercial luciferase test kit (BioOrbit-Wallac, Turku, Finland) and a 96 well plate luminometer (Amersham, Bucks).

MACROPHAGE ADHESION

Mononuclear cells were purified using commercial gravity separation media as described below. Fresh venous blood was obtained from a healthy 32 year old male volunteer. The blood was defibrinated by agitation in a vial containing sterile glass beads for 5 minutes. The blood was then diluted 1:1 with macrophage serum free medium (RPMI1640 medium, Gibco, Life Technology Ltd, Paisley) and slowly pipetted into a tube containing a purification medium (LymphoSep, ICN-Pharmaceuticals, Hants). The mixture was centrifuged at 400 *g* for 20 minutes to separate the cells. The central layer containing the mononuclear cells was sampled. The mononuclear cells were then pelleted by a further centrifugation at 400 *g* for 10 minutes. The mononuclear cells were resuspended in RPMI1640 medium and counted using a haemocytometer. Sample discs were placed in a 24 well tissue culture plate and 2×10^5 cells in 0.5 ml of media are pipetted onto each disc in sequence. The plates were incubated for 24 hours in a 37°C incubator under 5% carbon dioxide. The discs were then washed (×3) with PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. The fixed cells were then permeabilised by incubation with a 1% solution of Triton X100 (Sigma, Dorset) for 2 minutes, and macrophages detected using the protocol described below using an avidin-biotin-peroxidase kit (RapidStain, Sigma, Dorset).

Endogenous peroxidase activity in the cells was quenched by 72 hours' incubation in 9% hydrogen peroxide and repeatedly washed in PBS. Mouse anti-human macrophage (CD68) monoclonal antibody (Dako Ltd, Bucks, diluted 1:100 in PBS + 1% fetal calf serum) was added to each disc and allowed to incubate overnight at 4°C in a humidified chamber. The discs were then washed (×3) in PBS and macrophages observed using a high affinity biotin

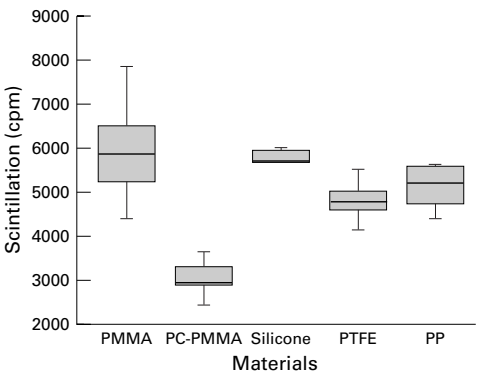

Figure 1 Adsorption of iodine-125 labelled fibrinogen to biomaterials after 2 hours' incubation in PBS.

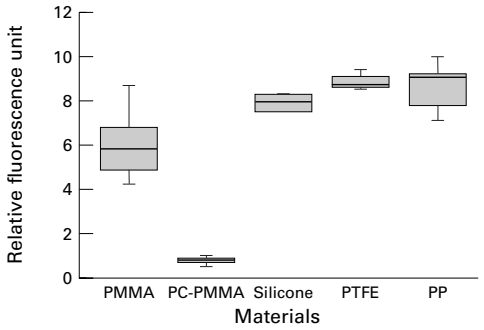

Figure 2 Adhesion of fibrin to biomaterials from fresh plasma after 2 hours' incubation.

conjugated anti-mouse antibody and an avidin-peroxidase detection system using AEC as a chromogen. The average number of macrophages (stained red) was determined in 50 randomly selected fields under light microscopy by a second "blind" observer.

DATA ANALYSIS

Results are presented graphically as box plots showing the 10th, 25th, 50th, 75th, and 90th centiles as vertical boxes with error bars. Statistical analysis was performed using SPSS for Windows software (SPSS Inc, Chicago, IL, USA). The Mann–Whitney non-parametric test was used to detect any statistically significant differences between uncoated PMMA and the other test materials.

Results

Fibrinogen adsorption is illustrated in Figure 1. Only PC coated PMMA discs reduced the amount of fibrinogen adsorption significantly ($p=0.004$), relative to PMMA. Figure 2 shows fibrin adhesion on the test materials. Fibrin adhesion to the PC coated PMMA discs was minimal, and significantly lower than uncoated PMMA ($p=0.004$), with other materials exhibiting similarly elevated fibrin adhesion levels.

PC coating of the PMMA discs also modified the cellular adhesion profiles and these results are shown in Figures 3 and 4. The reduction in human scleral fibroblast adhesion to PC coated PMMA was statistically significant ($p=0.002$) compared with the uncoated PMMA discs. The adhesion of human scleral fibroblast to silicone, polypropylene, and PTFE discs was not significantly reduced in comparison with PMMA (Fig 3). The adhesion of human macrophages was also significantly reduced by PC coating of PMMA ($p=0.01$) (Fig 4). Although both the PP and PTFE discs have a higher mean number of macrophages adherent per field in comparison with uncoated PMMA discs, these differences are not statistically significant ($p=0.057$).

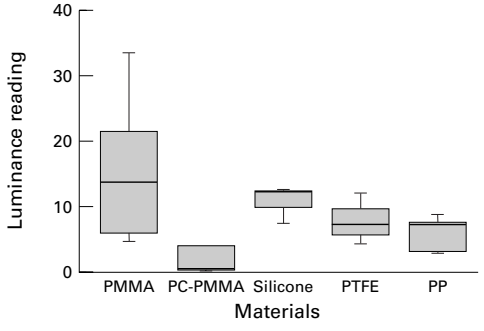

Figure 3 In vitro adhesion of human scleral fibroblast to biomaterials.

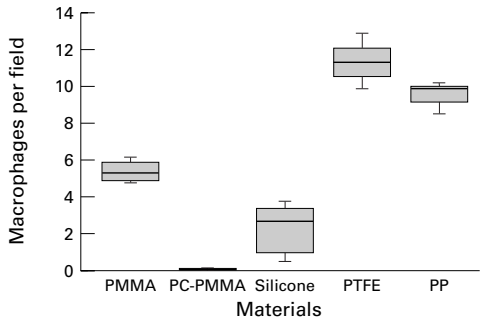

Figure 4 In vitro adhesion of macrophage to biomaterials.

Discussion

Implanted polymeric materials quickly adsorb proteins from the surrounding fluid or tissue environment. Adsorption occurs via a range of bonding mechanisms, and results in a biologically conditioned surface with modified interfacial properties. Cells interact predominantly with this modified surface rather than the material surface itself.[12] The biological adhesion characteristics of a material are therefore governed by its protein adsorption properties.

Fibrinogen adsorption may therefore be of particularly importance. In addition to its role as a fibrin precursor, fibrinogen appears to be pivotal in establishing inflammatory responses to implanted synthetic polymers.[13] Histological studies in complement or immunoglobulin depleted mice have revealed inflammatory responses similar to non-depleted controls.[14] In contrast, fibrinogen depleted mice did not mount an inflammatory response unless the test material was precoated with fibrinogen.[13] Fibrinogen is also thought to promote cell adhesion by integrin binding to an RGD motif similar to fibronectin.[15]

In our study, fibrinogen adhesion on PC coated PMMA was approximately half that seen for the other test materials. An indication of the possible importance of this reduction in fibrinogen binding is given by the results of the fibrin binding assay in which adhesion was virtually eliminated by the PC coating. Fibrinous occlusion is a common complication with all forms of GDDs. In clinical series, 8–11% of GDDs are occluded by fibrin in the early postoperative period.[4] Partial occlusion or significant fibrinous surface spoilation may be considerably more prevalent.

Partial occlusion is of particular relevance to GDDs in which small bore filtration channels are used as a resistance mechanism. From Poiseuille's formula, typical diameters required to regulate flow effectively at physiological aqueous outflow rates lie in the range 20–60 μm for single tubes up to 6 mm in length.[16] At these diameters, small accretions of biological debris could lead to relatively large percentage changes in cross sectional area and therefore flow performance.

In these studies, the PC coating also reduced cellular adhesion for both macrophages and scleral fibroblasts significantly. Differences between PC coated PMMA and other test materials were less dramatic than those observed in the protein adhesion assays. This relative reduction in effect may again be attributable to protein conditioning. Precoating with albumin makes material surfaces inert,[17] reducing subsequent adhesion of other protein and cellular elements. Albumin in the culture media used may have caused the material to become inert in our assays reducing cell adsorption to the uncoated materials, and this was demonstrated by the low level of macrophage adhesion to silicone discs. However, differences between PC coated and other test materials were significant none the less.

Beyond surface spoilation, macrophage adhesion in particular is thought to be an important initiator of chronic inflammatory responses to implanted foreign material.[12 18] Progressive fibrous encapsulation driven by a low grade foreign body inflammatory response limits the functional lifespan of contemporary GDDs.[4] Reducing the adhesion of fibroblasts and inflammatory cells may therefore significantly lengthen the functional life of GDDs.

The precise mechanism by which PC polymers resist non-specific protein adhesion is not well understood. Phosphorylcholine is zwitterionic, possessing both positive and negative electrical charges in close proximity, but is overall electrically neutral. The zwitterionic nature of the PC head group has a large hydration shell, and it is possible that this relatively large and stable surrounding barrier of water molecules mediates resistance to protein adhesion.

Other surface modification technologies, including heparin surface modification,[19] may also be effective in reducing surface spoilation. We restricted our comparison to materials used in contemporary GDDs. These materials (DMS, uncoated PMMA, PTFE, and PP) were significantly outperformed by PC coated PMMA in fibrinogen, fibrin, macrophage, and scleral fibroblast adhesion assays. PC coating is a new material technology that offers considerable promise in the field of GDD development.

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