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CD47-Dependent Molecular Mechanisms of Blood Outgrowth Endothelial Cell Attachment on Cholesterol-Modified Polyurethane

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Abstract

We previously showed that blood outgrowth endothelial cells (BOECs) had a high affinity for polyurethane (PU) covalently configured with cholesterol residues (PU-Chol). However, the molecular mechanisms responsible for this enhanced affinity were not determined. CD47, a multifunctional transmembrane glycoprotein involved in cellular attachment, can form a cholesterol-dependent complex with integrin $\alpha_v\beta_3$ and heterotrimeric G proteins. We tested herein the hypothesis that CD47, and the other components of the multi-molecular complex, enhance the attachment of BOECs to PU-Chol. Immunoprecipitation studies, of human and ovine BOECs, demonstrated that CD47 associates with integrin α_v and integrin β_3 as well as G $_{\alpha i-2}$ protein. The three-fold increase in BOEC attachment to PU-Chol, compared to unmodified PU, was reversed with the addition of blocking antibodies specific for CD47 and integrin α_v and integrin β_3 . Similar results were observed with the addition of methyl-beta-cyclodextrin (M β CD), a known disruptor of the CD47 complex as well as of the membrane cholesterol content, to seeded BOEC or PU-Chol films. Reducing CD47 expression, via lentivirus transduced shRNA, decreased BOEC binding to PU-Chol by 50% compared to control groups. These data are the first demonstration of a role for the CD47 cholesterol-dependent signaling complex in BOEC attachment onto synthetic surfaces.

1. Introduction

Seeding autologous endothelial cells on synthetic surfaces is a common strategy to reduce inflammation, thrombosis, and ectopic calcification of implantable cardiac devices. The recent identification and characterization of blood outgrowth endothelial cells (BOECs), which are the progeny of a marrow derived, transplantable, circulating endothelial progenitor cell, has heightened interest in this approach by suggesting that a population of rapidly dividing endothelial cells can be easily acquired from peripheral blood [1,2]. We and others have demonstrated the feasibility of using BOECs to replace the function of an intact endothelium

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on implanted biomaterials [3–6]. Problems such as cell retention and clearance by the host immune system all reduce the efficacy of seeding BOECs, or any other progenitor cell, on to synthetic surfaces with the intention of improving the biocompatibility of vascular devices [7, 8]. Central to addressing these issues is achieving a better understanding of the molecular mechanisms involved in BOEC attachment to modified synthetic surfaces. Such information would be useful in designing second generation synthetic surfaces capable of enhanced endothelial cell adhesion.

Polyurethane elastomers (PU) are commonly utilized synthetic biomaterials, both clinically and experimentally, in various medical devices such as heart valves, pacemaker leads, and left ventricular assist devices. Unfortunately, due to thrombosis, calcification, and biodegradation, device failure is commonly reported [9–12]. A number of novel alterations to PU has been attempted to alleviate these problems. Our group synthesized and characterized a bulk-modified PU configured with mercapto-cholesterol (PU-Chol) via bromoalkylation of hard segment urethane nitrogen [4,13]. Analysis of the physical properties of PU-Chol films revealed that the addition of cholesterol moieties to PU increased the surface hydrophobicity and decreased the surface roughness [4]. In addition, we demonstrated superior attachment and retention of BOECs on PU-Chol surfaces than on unmodified PU [4,13]. Pulmonary valve leaflets composed of PU-Chol, seeded with autologous BOECs showed superior cellular retention and reduced thrombogenicity compared to unmodified PU valve leaflets in a sheep model [13]. Although these earlier studies clearly demonstrated the superior cell adhesion and retention properties of PU-Chol, compared to unmodified PU, the molecular mechanisms contributing to these observations were not identified.

CD47, also known as integrin-associated protein, is a membrane spanning glycoprotein, originally isolated with integrin $\alpha_v\beta_3$ which functions as an intercellular signaling molecule and as an extracellular ligand for myeloid cells. CD47 forms a multiprotein complex with the integrin $\alpha_v\beta_3$ and heterotrimeric G proteins that requires membrane cholesterol to maintain its integrity and function [14,15]. Although integrin $\alpha_v\beta_3$ mediated adhesion and cell spreading appears normal in tested CD47 deficient cells, CD47 is necessary for certain $\alpha_v\beta_3$ mediated signaling events in which the presence of cholesterol appears to be essential [15].

In our current work, we have examined the role of exogenous cholesterol on the CD47 multi-molecular complex with respect to the attachment of BOECs on PU surfaces. Our working hypothesis was that the surface cholesterol on PU-Chol films can interact with the cellular CD47 complex to improve BOEC adhesion. The goals of this study were 1) to examine the roles of cellular cholesterol and PU bound cholesterol on BOEC attachment, and 2) to assess the involvement of the CD47 multi-molecular complex in the process, focusing mostly on the role of CD47 itself, since it is known to exert its effects through the function of integrins [16] and the G proteins [14,15].

2. Materials and methods

2.1. Materials

The PU used was Tecothane TT1074A (Thermedics, Waltham, MA), a polyether polyurethane. A mouse monoclonal antibody raised against human CD47 (B6H12) was purchased from BD Pharmingen (Franklin Lakes, NJ). A mouse monoclonal antibody directed against human integrin α_v (LM142) was purchased from (Chemicon International, Billerica, MA). A mouse monoclonal antibody directed against human integrin $\alpha_v\beta_3$ (23C6), a goat polyclonal antibody raised against human integrin β_3 (N-20), and rabbit polyclonal antibodies against G $_{\alpha i-2}$ (T-19) or ERK-2 (C-14) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Hexadimethrine bromide, methyl-beta-cyclodextrin (M β CD), human CD47 shRNA lentiviral particles and Puromycin were purchased from Sigma (St. Louis, MO). EGM-2 media was

acquired from Lonza Clonetics (Basel, Switzerland). DAPI was purchased from Vector Laboratories, Inc. (Burlingame, CA). Tween 20 and sodium dodecylsulfate-polyacrylamide electrophoresis gels were purchased from Bio-Rad (Hercules, CA). The enhanced chemiluminescence detection system, a product of Amersham, GE Healthcare (Piscataway, NJ), was used along with X-ray films (Danville Scientific).

2.2. Polyurethane and cholesterol-modified polyurethane (PU-Chol)

PU-Chol was synthesized by bromoalkylation of the urethane nitrogens followed by reactive attachment of mercaptocholesterol as previously described [4]. Both PU and PU-Chol were dissolved in dimethylacetamide, and then were solvent cast as films with thickness ranging between 159 and 220 μm as used in prior studies [4,13]. For attachment experiments, they were cut into appropriate sizes for cell culture wells.

2.3. Cells and cell culture

As indicated, BOECs of human (hBOEC) or ovine (oBOECs) origin were cultured in 100-mm tissue culture-treated dishes as reported [4], and grown in EGM-2 medium. The culture medium was changed every 2–3 days. The cells used in the experiments were of passages between seven and twelve. Both hBOECs and oBOECs were characterized as previously described [2,4,17]. Markers such as von Willebrand's factor (VWF), Flk-1, P1H12, VE-cadherin, and uptake of acetylated low-density-lipoprotein were used to ascertain endothelial phenotype [2,17].

2.4. Shear Adhesion Studies

Shear adhesion studies were performed as previously noted [4,13]. BOECs of human origin were grown to confluence on microscope slides (73×38×1 mm) coated with PU or PU-Chol. The cell containing slides were inserted into a parallel plate flow chamber whereupon non-pulsatile, laminar shear flow was delivered using culture medium heated to 37°C. Shear was maintained at 75 dynes/cm² for 2 hours. At the end of the protocol, slides were detached from the chamber, washed in phosphate buffered saline and then fixed with 4% paraformaldehyde. Cells were stained with DAPI and quantified as the number of DAPI-positive cells in a minimum of 20 random, 200× fields.

2.5. Immunoprecipitation with anti-CD47 antibody and Western Blot analysis

In order to assess the presence of the CD47 multi-molecular complex, immunoprecipitates with anti-CD47 antibody were obtained. Cultured ovine or human BOECs were washed three times with PBS and scraped in ice-cold lysis buffer (100 mM pH 8.1 Tris-HCl, 10 mM EDTA, 1% Triton X-114, a proportioned amount of complete protease inhibitor cocktail (Roche), 0.2 mM orthovanadate). The cellular lysates were passed through a 21-gauge needle, and the proteins were quantified using micro BCA protein assay kit. Prior to immunoprecipitation, the oBOEC lysates were spun down at 10,000 g for 10 minutes. The collected supernatant was first incubated with 5 μg of anti-CD47 antibody (B6H12) for an hour and then, incubated with the corresponding amounts of immunoaffinity agarose beads (Calbiochem) on a rotator rack at 4°C overnight. The immunoprecipitated CD47 complex proteins were resolved on a 4–15% gradient sodium dodecylsulfate-polyacrylamide electrophoresis gel using the method described by Laemmli [18], and the proteins were transferred to a 0.2 μm pore size polyvinylidene fluoride (PVDF) membrane (Invitrogen), followed by immunoblotting for the associated complex components using respective antibodies, integrin α_v , integrin $\alpha_v\beta_3$, integrin β_3 (N-20), and G_{ai-2} (T-19) at manufacturers' recommended dilutions in 10 mM pH 7.5 Tris-HCl, 100 mM NaCl, and 0.1 % Tween 20 (TTBS) with 5% non-fat milk. The immune complexes were detected with the species-appropriate, horseradish peroxidase-conjugated secondary antibodies in recommended dilutions in TTBS with 5% non-fat milk and were visualized with an enhanced chemiluminescence detection system on X-ray films.

2.6. Attachment assays

Cell attachment assays were conducted as in our previous studies [4] with some modifications. In brief, duplicate samples of PU or PU-Chol films were cut into 1cm² sections and placed on the bottom of a 24-well plate. Ovine BOECs were trypsinized and preincubated for a pre-determined time period in the medium with or without various interfering agents prior to seeding into each well (100,000 cells/well). The seeded plate was incubated at 37°C. At timed endpoints (15 and 30 minutes), the films were washed with PBS X3, and adherent cells were fixed with cold 4% paraformaldehyde. Cell quantification was performed by staining with DAPI, a nucleus-specific fluorescent stain, and fifteen random fields per each film piece were selected for nucleus counting under 200× magnification with the appropriate fluorescent filter set using a Nikon TE-300 inverted microscope (Nikon, Inc., Tokyo, Japan).

2.7. Cholesterol modulation experiments

The contribution of cholesterol was investigated by an addition of M β CD, a known disruptor of the CD47 integrin complex [15] as well as of the membrane cholesterol. The cells were pre-incubated with or without 10 mM [19] of M β CD for 15 minutes at room temperature on a plate rocker prior to seeding on PU or PU-Chol as described above. To investigate the effect of M β CD on PU surfaces, PU and PU-Chol films were pre-treated with 10 mM M β CD overnight, and they were washed with PBS prior to conducting attachment assays.

2.8. Assessment of the contribution of the components of the CD47 multi-molecular complex

Blocking agents against the components of the CD47 multi-molecular complex were selected for attachment experiments to evaluate their contribution in the process (20 μ g/ml of anti-human CD47 antibody, 100 μ g/ml of RGD peptide against human integrin $\alpha_v\beta_3$ and 20 μ l/ml of mouse non-specific IgG (Thermo Scientific) as the control blocking agent. Prior to seeding, oBOECs were incubated with the agents for 30 minutes on a rotating rocker at 4°C.

2.9. CD47 knock-down with CD47 shRNA lentiviral particle transduction

The short hairpin RNA (shRNA) targeted to the mRNA which translates to the cytoplasmic region of human CD47, and has 85% homology with sheep CD47 (TRC# TRCN0000007836), was used at a multiplicity of infection (MOI) of 5 to transform oBOECs according to manufacturer's instructions. Briefly, 50,000 cells of oBOEC were plated in a well of a 24-well-plate on day 1. On day 2, the cells were primed with 8 μ g/ml of hexadimethrine bromide, and a pre-determined amount of viral particles were added to the culture. After 24 hours of transduction, the virus-containing medium was replaced with fresh, complete medium. Puromycin (2 μ g/ml) selection was initiated on the following day and surviving colonies were expanded for experiments. The volume of transduction viral particles was calculated using the formula: (# cell)/(desired MOI)/(lentiviral particle concentration (TU/ml)). GFP-lentiviral particles transduced oBOECs were used as the control for comparison (kindly provided by Dr. Philip Zoltick, Children's Hospital of Philadelphia).

2.10. Assessment of CD47 shRNA knock-down in oBOECs and attachment assays

The status of the CD47 shRNA knock-down was first assessed by standard Western blotting techniques using anti-CD47 antibody. The same membrane was re-probed with anti-ERK-2 antibody (C-14) for normalization of the protein expression. The degree of knock-down was quantified using the obtained films and ImageJ software. CD47 shRNA transduced BOEC attachment assays were performed as previously described using non-transduced and GFP-lentiviral particle transduced oBOECs as the control for comparison.

2.11. Statistical Analysis

The results were analyzed using ANOVA on Ranks Kruskal-Wallis Test with Tukey post-hoc method, and the value of $p < 0.05$ was used as showing a statistical significance.

3. Results

3.1. Disruption of cholesterol with β -cyclodextrin

To determine the contribution of membrane cholesterol upon BOEC attachment to modified and cholesterol-modified surface, we pretreated the cells with a known disrupter of membrane cholesterol M β CD [20,21]. As previously observed [4], there was nearly a three-fold increase in attachment of oBOECs on PU-Chol compared to unmodified PU. However, a treatment of the BOEC with 10 mM M β CD significantly ($p < 0.001$) reduced their attachment to PU-Chol (67%) compared to a modest and not statistically significant decrease (34%) seen on unmodified PU (Figure 1A). However, the treated cells were able to regain their adherent capacity to the baseline within 30 minutes once M β CD was eliminated from the medium, indicating that the cells were not harmed irreversibly with the M β CD treatment (Results not shown).

To determine the contribution of the cholesterol that is incorporated into the PU as a result of our chemical modification, we incubated PU-Chol films overnight with M β CD (Figure 1B) prior to seeding. Control films were incubated with PBS. Ovine BOEC attachment to the PU-Chol was assessed in the presence and absence of M β CD. As seen in Figure 1B, preincubating the PU-Chol films with M β CD decreased oBOEC binding by 50%. The attachment of oBOECs to PU-Chol films was further inhibited by the presence of M β CD in the cell media. These results confirm a critical role, with respect to BOEC attachment, for the cholesterol moieties in PU-Chol.

3.2. Adhesion of Human BOECs under high shear

There are many potential clinical applications of BOEC based therapeutic strategies. Our previous results showed that oBOECs could be used to seed PU-Chol heart valve leaflets [13]. To begin to relate these earlier results for a potential clinical application, hBOECs were cultivated on microscope slides coated with either PU-Chol or control PU. Cell culture media was passed over the BOECs at valvular levels of shear force (75 dynes/cm²) for two hours. At the end of the protocol, retained cells were visualized using phase contrast microscopy. Figure 2A shows robust cell retention observed on PU-Chol seeded with hBOECs. In contrast, hBOECs seeded on unmodified PU coated slides were largely removed from the PU coated slide. The extent of cell retention was determined via counting DAPI-stained nuclei. As seen in Figure 2B, hBOEC retention on PU-Chol coated slides was almost 8-fold greater compared to unmodified PU coated slides.

3.3. CD47 colocalization studies

The formation of the CD47, integrin $\alpha_v\beta_3$ and heterotrimeric G-protein complex has not been confirmed in BOECs as it has been in other cell types [14]. To ascertain if CD47 expressed on BOEC associates with the integrin heterotrimeric protein complex we immunoprecipitated CD47, and any associated proteins, and probed for the known protein components in BOECs from either ovine or human origin. Figure 3 shows representative Western blot analysis of resolved immunoprecipitated CD47 associated proteins. In both oBOEC (Figure 3A) and hBOEC (Figure 3B) lysates, CD47 colocalized with integrin α_v , integrin β_3 and the G $_{\alpha i-2}$ protein subunit of the heterotrimeric G-proteins. Of note, the commercially available antibody directed against the human $\alpha_v\beta_3$ integrin heterodimer did not react with the ovine homologue (data not shown). These data are consistent with results observed in other cell types and

identified reagents for function blocking studies to further determine the role of CD47 and its associated proteins in mediating BOEC attachment to PU-Chol [14,22].

3.4. CD47-integrin $\alpha_v\beta_3$ antagonism

As previously reported by others, blocking antibodies [16,23] and RGD peptides [16,23] can inhibit the CD47-integrin $\alpha_v\beta_3$ mediated signaling pathway. To determine the contribution of this multi-molecular complex upon BOEC attachment to PU-Chol surfaces, we incubated oBOECs (Figure 4A) in the presence of anti-CD47 antibody or in the presence of molecular antagonists to G protein coupled receptors or integrin $\alpha_v\beta_3$. The presence of anti-CD47 antibody significantly reduced the number of adherent oBOECs on PU-Chol; compared to the non-treated and non-specific IgG-treated cells. Addition of the blocking agent relevant to integrin $\alpha_v\beta_3$ (RGD peptide) also significantly decreased the number of adherent oBOECs on PU-Chol. Notably, the agents tested here did not alter the attachment of oBOECs on unmodified PU. Attachment of hBOECs to PU-Chol was also significantly inhibited in the presence of antibodies directed against CD47 or the integrin $\alpha_v\beta_3$ dimer. Although these antibodies did not significantly affect binding of hBOECs to unmodified PU, binding of hBOECs to unmodified PU was markedly higher compared to oBOECs.

3.5. CD47 shRNA

To further assess the role of CD47 in BOEC attachment, we established, via CD47 shRNA, a population of oBOECs that expressed reduced levels of CD47 (oBOEC^{CD47-}). Western blot analysis was used to confirm the decrease in CD47 protein expression in the CD47 lentivirus transduced oBOECs (Figure 5A). This was quantified as $30 \pm 2.7\%$ reduction through normalization with the expression of nonphosphorylated ERK-2 protein. To further assess the role of CD47 upon BOEC attachment to cholesterol modified PU, we performed attachment assays using oBOEC^{CD47-}. As shown in Figure 5B, oBOEC^{CD47-} had a reduced capacity to bind to PU-Chol by about 50% compared to non-transduced or GFP lentivirus transduced oBOECs after 30 minutes of seeding. However, their ability to bind unmodified PU was not affected.

4. Discussion

Given that BOECs represent an easily acquired source of autologous endothelial cells and their potential for clinical applications, BOECs are an important cell type for investigating cellular attachment mechanisms to biomaterial surfaces. Others and we have shown that biomaterials can be modified to increase BOEC attachment and retention [3,5,6,13]. In spite of their potential clinical importance, little is known of the molecular mechanisms that regulate BOEC physiology. In this paper we identified a novel role for CD47, a ubiquitously expressed cell surface receptor, in the attachment mechanism of BOECs to PU modified with cholesterol moieties. In addition these data begin to identify the molecular mechanism responsible for the increased BOEC binding to our novel cholesterol modified polyurethane.

We have previously reported that covalently linking cholesterol to polyurethane hard segments can increase the attachment rate and cell retention, under physiological shear forces, of BOECs and vascular endothelial cells [4,13]. This observation was not completely unexpected, as hydrophobic surfaces, such as those resulting from the cholesterol modification, have demonstrated enhanced cell retention compared to more hydrophilic surfaces [24–26]. To begin to understand the underlying molecular mechanisms influencing BOEC binding to synthetic surfaces, we focused on discerning the molecular mechanisms involved in BOEC attachment to PU-Chol. In our previous studies we observed that the maximal endothelial cell attachment to PU-Chol, compared to unmodified PU was achieved at thirty minutes post-

seeding [4]. Hence, in these current investigations, we examined the roles of CD47 and cholesterol related mechanisms upon BOEC attachment at thirty-minutes.

Appending cholesterol to synthetic surfaces by our group has been shown to enhance cell attachment [4,13]. Of course cholesterol is a vital component of the cell membrane where it functions to maintain membrane integrity and to sequester membrane proteins. Work by others has shown that CD47 can form a cholesterol dependent multimeric protein complex with integrin $\alpha_v\beta_3$ and the $G\alpha_i$ protein [15,16]. Given the influence of cholesterol upon enhanced BOEC binding to PU that we had reported previously [4,13], CD47 was identified as a molecule of interest in identifying the attachment mechanisms responsible for enhanced binding to PU-Chol. However, CD47 also has broadly defined functions independent of the previously mentioned integrin-signaling complex. These additional roles of CD47 do not require cholesterol and are responsible for immune recognition as well as fibroblast migration and aggregation [14,27].

The results observed with the use of M β CD clearly demonstrate the significance of cholesterol in the previously observed enhanced oBOEC attachment on PU-Chol. The presence of M β CD significantly altered the attachment of oBOECs to PU-Chol, but not to unmodified PU. It probably disrupted the cholesterol-dependent CD47 multi-molecular complex as well as hydrophobic interactions and other pathways. We further assessed the contribution of appended cholesterol in the bulk modified PU-Chol by a pre-treatment of PU-Chol with M β CD prior to oBOEC seeding. Very interestingly, oBOEC attachment to PU-Chol was diminished with the pre-treatment, indicating that the surface oriented cholesterol in the bulk modified PU-Chol contributes to the attachment of BOECs to the PU surface. It is plausible that M β CD molecules sequester cholesterol molecules, becoming inaccessible for BOEC interactions even after washing with PBS, leading to the reduced cell attachment.

The sheep was our *in vivo* model in which we showed persistent BOEC seeding of PU-Chol pulmonary heart valve leaflets beyond 90 days implantation [13]. As such, we used oBOECs to identify CD47 dependent attachment mechanisms. However, the extracellular region of CD47 has a high level of sequence variation between species and compatible reagents for ovine CD47 have not been completely characterized. Therefore, we used human BOECs to confirm our oBOEC observations. The use of blocking antibodies directed against CD47 and integrin $\alpha_v\beta_3$ showed that these protein components were important in BOEC attachment to the PU-Chol film, but not to the unmodified PU. It was surprising that hBOEC attachment to unmodified PU was almost as robust as the attachment observed with hBOEC attachment to PU-Chol. Whether this is a result of variability between individual BOEC samples or if there actually is species variability in BOEC attachment to unmodified PU remains to be determined. As shown above, CD47 had a significant role in the attachment of both oBOEC and hBOEC, strongly suggesting a conserved CD47 attachment mechanism in both species.

We used an shRNA strategy to further confirm the role of CD47 in attachment to PU-Chol. We chose to use ovine BOECs for these investigations since sequence comparisons showed an 85% homology between the ovine and human genome in this region, and reduced gene expression was confirmed using Western blot analysis. Our BOEC^{CD47-} experiments showed that attachment of these cells to the PU-Chol surface was reduced by half compared to control cells. These numbers corresponded well with the reduction in CD47 expression. It was also clear that targeting CD47 or integrin $\alpha_v\beta_3$, via blocking antibodies as well as reducing CD47 expression via shRNA was not sufficient to completely block BOEC attachment to the PU-Chol surface. These results strongly suggest the existence of additional molecular pathways that contribute to BOEC attachment to PU-Chol.

CD47 has been demonstrated to have roles in both cell adhesion and immune evasion [14]. Our current application of BOEC has been to seed autologous cells onto cholesterol-modified polyurethane [13]. Thus, this study did not examine the role of CD47 in down regulating the immune response to implanted BOEC seeded materials. However, we have identified CD47 as an important molecular component in BOEC attachment to cholesterol-modified polyurethane. Further investigations into CD47 mediated signaling events in BOEC may assist in the development of future biomaterials that can both enhance BOEC attachment as well as reducing inflammatory responses to implanted biomaterials.

5. Conclusions

These studies have demonstrated an essential role of CD47 in BOEC attachment to cholesterol modified PU surfaces. We have shown that the cholesterol dependent CD47 multi-molecular complex along with the presence of immobilized cholesterol on the modified polyurethane is required for enhanced BOEC attachment to PU-Chol surfaces. These data also strongly suggest that additional molecular mechanisms, independent of CD47, also contribute to BOEC attachment.

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Figure 1.

Cellular and Polyurethane Immobilized Cholesterol are critical for BOEC attachment to Polyurethane surfaces. (A) Ovine BOECs were incubated with 10 mM of MβCD, a known disrupter of membrane cholesterol, and seeded on unmodified or cholesterol modified polyurethane films. BOEC attachment was assessed after 30 minutes as detailed in *Materials and Methods*. As shown, the presence of MβCD (white bars) in the medium significantly decreased oBOEC attachment to PU-Chol. (B) PU-Chol films were incubated overnight in the presence of phosphate buffered saline or PBS containing 10 mM of MβCD, as detailed in *Materials and Methods*. Cultured oBOEC cells were seeded on the PU-Chol films in the presence of absence of MβsCD. Cell attachment was assessed at 15 and 30 minutes.

Preincubating the PU-Chol films (white bar) with M β CD significantly reduced BOEC attachment compared to non-M β CD treated films (black bar). Maintaining a presence of M β CD in the BOEC culture media further reduced attachment (grey bar). Differences in the cell attachment to the cholesterol modified polyurethane surfaces between experiments A and B are attributed to the overnight incubation of the PU-Chol film with PBS in experiment B. Values are expressed as average $\pm$ standard error of the mean (n = 20). Significance was determined using ANOVA.

Figure 2.

Adhesion of human blood outgrowth endothelial cells exposed to laminar flow: (A) Representative photomicrographs of hBOECs grown on either polyurethane (PU) or polyurethane modified with cholesterol (PU-Chol) coated microscope slides and exposed to 75 dynes/cm² of shear flow. Magnification 200×. (B) Graphical representation of hBOEC retention after 2-hour exposure of simulate valvular levels of shear (75 dynes/cm²). Significantly more hBOECs were retained on PU-Chol surfaces following shear flow than on unmodified PU coated slides.

Figure 3.

Representative Western Blot analyses of CD47 immunoprecipitates probed for integrin $\alpha_v\beta_3$ and a G-protein coupled receptor. Cell lysates from BOECs of ovine (A) or human (B) origin were immunoprecipitated with anti-CD47 antibody as detailed in Materials and Methods. Resolved proteins were probed for the presence of integrin $\alpha_v\beta_3$ and the α_i subunit of the G-protein coupled receptor. In both sheep and human BOEC, integrin $\alpha_v\beta_3$ and α_i colocalized with CD47.

A. **B.** **Figure 4.**

Targeting the CD47, integrin $\alpha_v\beta_3$ multimolecular complex disrupts BOEC attachment to PU-Chol but not to unmodified PU films. BOECs of either ovine (A) or human (B) origin were cultured on PU or PU-Chol films for 30 minutes in the presence of inhibitors to the CD47-integrin $\alpha_v\beta_3$ G protein signaling pathway. Films were assessed for BOEC attachment after 30 minutes as detailed in *Materials and Methods*. The function blocking antibody to CD47 significantly reduced BOEC adhesion. Likewise the tripeptide (RGD) also blocked BOEC attachment. In contrast, non-specific IgG had no effect upon BOEC attachment to PU-Chol. Values are expressed as average $\pm$ standard error of the mean (n = 20).

Figure 5. Reduced CD47 expression inhibits BOEC binding to PU-Chol. CD47 expression was diminished in ovine BOECs by CD47 shRNA, via a lentiviral vector. Western blot analysis (**A**) was used to confirm reduced CD47 expression. Comparison was made to the expression of ERK-2 protein. (**B**) Adhesion to PU-Chol was significantly reduced in CD47 shRNA expressing oBOEC compared to oBOECs expressing GFP.