Adhesion of microvascular endothelial cells to metallic implant surfaces

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The objective of this study was to explore the molecular mechanisms of adhesion of endothelial cells (ECs) to implant grades of titanium alloy (Ti) and stainless steel (SS), compared to tissue culture polystyrene (PS). The idea is that promotion of EC adhesion to implant surfaces during the initial stages of healing may be critical in the formation of a capillary bed intimately associated with the implant surface. Ultimately this could be expected in turn to promote bone formation close to the surface and a more stable implant/bone interface. Surfaces were coated with either peak 1 fibrinogen $\gamma A\gamma A$, fibrinogen Fr I-9, fibrinogen fragment D1, fibronectin, vitronectin, or fetal calf serum and then post-coated with bovine serum albumin (BSA) to block non-specific cell adhesion. Surfaces with BSA alone and no other protein coating were also evaluated. Fibronectin coating maximized cell adhesion on all three surfaces, and adhesion was highest on PS. BSA blocked cell adhesion to PS (and most adhesion to SS) much better than to Ti. These results provide evidence that BSA adsorption on the metal surface is unable to effectively block the adhesion of the cells to the Ti. These data may provide a basis for understanding *in vivo* observations that soft tissue becomes attached to a Ti surface more rapidly and with more bone formation than to SS. Evidence is also presented that $\alpha_v\beta_3$ plays an important role in adhesion of ECs to the Ti surface. These experiments also provide preliminary data which may reflect some of the features of initial EC adhesion to metal implants.

1. Introduction

The clinical success of orthopaedic implants depends in part on the cellular response in the immediate vicinity of the implant. The functional states of adhesive molecules and the dynamics of cell adhesion are important in understanding the cellular response to implants. In this response, there is extensive cell migration which most likely involves extracellular matrix/integrin adhesion mechanisms. There is also a general consensus that adhesion of involved cell to implant surfaces is likely to encourage the formation of an intimate stable implant/bone interface. In this connection, much of the prior *in vitro* research has described the adhesion of osteoblasts, osteoclasts or osteosarcoma cells to implanted metals, hydroxyapatite and bone [1–8]. Also much of the prior research has concentrated on surface characteristics of metals rather than on identifying the adhesion receptor mechanisms of the adherent cells [9, 10]. The rationale underlying the work described here is that the initial events required for successful establishment of the implant also include adhesion of ECs to the implant surface. The basis for this idea is described below.

1.1. The role of endothelial cells

The importance of ECs in bone formation has been recognized for many years. In 1927, Sir Arthur Keith [11] stated that ''Cells which assume a bone-forming role are derived from the endothelium of the capillary system''. Almost 40 years later, Trueta [12] again stressed the importance of vessels in osteogenesis. It has also been observed that the first non-inflammatory cells present next to the implant are fibroblast and ECs [13]. More recently it has been shown that endothelial cells play an important role in the delivery of cells to the inflammation site, chemotaxis, cell adhesion and extravasation [14–16]. Cytokines in tissues adjacent to the implant have been shown to induce EC adhesion molecules [17, 18]. ECs may also have a role in clot retraction and the tissue response to wear products around orthopaedic implants [19, 20].

Also, ECs (as well as fibrinogen and its fragments) play an important role in healing around the implant. The implant is essentially put into a blood clot, which is formed by fibrin that is degraded in a complex sequential manner during fibrinolysis. The vascular endothelium is

a focal point for the interactions of the coagulation and fibrinolytic enzyme pathways [21]. ECs play a key role in the fibrinolytic pathway through several direct and indirect mechanisms [21]. The process of angiogenesis is of primary importance during the initial healing process.

Characterizing the cellular responses involved in angiogenesis and bone formation adjacent to the implants is critical to understanding and promoting implant biocompatibility and improving stable fixation of implants, especially implants that replace joints. Rather than simply maintaining the alignment of a fracture while it is healing, cellular adherence and mitotic growth are essential for the formation of new capillaries adjacent to the implant. These capillaries deliver nutrients, growth factors, osteoprogenitor cells and other materials which are needed for local bone formation. Thus our research focused on ECs because of their pivotal role in osteogenesis.

Human umbilical vein ECs (HUVECs) have frequently been used as a model in EC adhesion studies due to the ease of access to these cells . Albelda *et al*. [26] have reported that EC integrin expression may not differ between ECs from different parts of the body, nor is the integrin composition modified by the length of time in culture. However, in our study, human microvascular (capillary) ECs were utilized since they, rather than HUVECs, may more closely approximate the cells that are present *in vivo* adjacent to the implant.

1.2. Role of adhesive proteins

The specific aim of this research is to characterize the adhesion of ECs on metal implant surfaces coated with different adhesive proteins. The function of adhesive molecules and the dynamics of cell adhesion are important in understanding the cellular response to implants. In this response, there is extensive cell migration which most likely involves extracellular matrix/integrin adhesion mechanisms [27]. Ideally, the area around the implant should be compatible with the ingrowth of ECs to enhance the cellular response that promotes healing and leads to a stable implant fixation.

It has recently been shown that an osteosarcoma cell line (which has many osteoblast characteristics) will adhere to titanium via integrin mediated adhesion, however there are no reports concerning ECs [3]. Also it is not clear that the *in vitro* binding of cells directly to implant biomaterials via integrins is relevant to the *in vivo* situation. The implant *in vivo* is probably first coated with plasma proteins and blood coagulation materials. Some of the matrix proteins found during inflammation and wound healing are fibronectin, fibrin(ogen) and vitronectin [28]. The invading ECs probably first encounter fibrinolytic fragments and plasma proteins on the surface of the implant. Therefore, maximizing the adhesion of ECs in the presence of plasma, platelets, fibrinogen and fibrin fragments may provide a more appropriate system in which to investigate the roles of the molecules involved and the mechanisms by which they act.

Fibrinogen, fibronectin, vitronectin as well as other proteins not used in this study (collagen, osteopontin and thrombospondin) are extracellular proteins which are known to have important roles in regulating cell function by signal transduction mediated through specific cell surface receptors. These proteins have important critical roles in processes vital to proper wound healing such as angiogenesis. The proteins utilized in this study to coat the implant surfaces were: (1) fibronectin (Fn), a protein found in many extracellular matrices (and in plasma), which is vital in response to injury and wound healing [29], (2) vitronectin (Vn), a blood plasma protein that induces cell spreading and migration and has an effect on cell growth and differentiation in cells expressing $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_{IIb}\beta_3$ integrins [30], (3) peak 1 fibrinogen $\gamma A\gamma A$, a soluble plasma protein important in fibrin formation and blood coagulation (also, the fibrinolytic products are important in endothelial cell migration during healing), (4) albumin, the most abundant protein found in plasma and, (5) serum which contains many proteins, including some of which are the first to adhere to the surface of an implant along with fibrinogen.

1.3. Role of integrins

Recently, a variety of studies have examined the role of integrin receptors in the cellular response to orthopaedic implant materials [2, 3, 5, 7, 8, 31]. The studies concerning attachment characteristics of bone cells via their receptors to various metal surfaces have added a great deal to our understanding of the mechanisms surrounding bone repair and implant biocompatibility. It is our assumption that the attachment characteristics of ECs are also vitally important in these activities.

Various monoclonal antibodies (Mabs) and peptides, which bind to important EC integrins or integrin subunits, were also used to facilitate identification of the integrins which mediate microvascular EC adhesion to the SS and titanium alloy discs.

2. Experimental procedures

2.1. Metal discs

Discs of standard implant grades of Ti (Ti-6Al-4V) and SS (22-13-5) were prepared to fit tightly at the bottom of the wells of Immunolon 1™ 96 well polystyrene (PS) tissue culture plates (Dynatech Laboratories Inc., Shantilly, VA, USA). We had previously found that ECs settled to the metal surface with minimal or no adhesion to the sides of the well. The metal surface finish was quantified using a motor driven surface roughness gage (Pocket Surf® Federal Products Corp., Providence, RI, USA). The metals were cut from rod stock by lathe turning and the diametric path of the discs had the highest roughness. The average roughness $\pm$ standard deviation for the Ti discs (diametric path) was 0.90 ± 0.28 μRa, and for SS 0.66 ± 0.16 μRa. The metal surfaces were cleaned by sonication in a warm alkaline detergent solution, rinsed in deionized water, passivated in 30% nitric acid, washed in deionized, sterile distilled (ultrapure) water and sterilized under a UV lamp inside a tissue culture laminar flow hood. Implant grade cast cobalt alloy surfaces would have been more clinically relevant than SS, but cobalt alloy discs were not available for this study.

2.2. Cell culture

Human microvascular ECs (Clonetics Corporation, San Diego, CA, USA) were grown in endothelial cell growth medium containing 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml of gentamicin, 50 ng/ml amphotericin-B, 3 mg/ml bovine brain extract, and 6% fetal bovine serum. This cell line was chosen for this preliminary study because it was the most relevant cell line which was also easy to obtain and grow in culture with very little cost and effort compared to isolating our own primary cultures. Although the cell line is not of bone origin, it is human microvascular and therefore should be very similar to human bone microvascular cells. The cells were grown to 80–95% confluence and then trypsinized (2.5 mg/ml trypsin, Sigma Diagnostics, St. Louis, MO) and gently scraped for release from the flask, or a 0.025% trypsin/0.01% EDTA solution (Clonetics) was used to release the cells from the surface of the flask after which trypsin neutralization solution (Clonetics) was added. The cell suspension was centrifuged at 220 × g for 5 min, the supernatant liquid decanted and the cells suspended in fresh endothelial cell growth medium. The suspended cells were allowed to recover from trypsinization by incubation in fresh endothelial growth medium to 30 min at 37 °C. The cell suspension was then centrifuged, decanted and washed with endothelial basal medium, which did not contain bovine brain extract or fetal bovine serum.

2.3. Cell adhesion assay

Fibrinogen peak 1 ($\gamma A\gamma A$) (10 μg/ml), fibrinogen fraction I–9 (10 μg/ml), fibrinogen fragment D1 (10 μg/ml), fibronectin (10 μg/ml), vitronectin (1 μg/ml) and fetal bovine serum were incubated in individual wells (100 μl/well) overnight at 4 °C in phosphate buffered saline (Sigma). Wells were post coated for one hour at room temperature with 0.5% bovine serum albumin (BSA) (100 μl/well) prior to addition of the cells in order to block nonspecific binding. Wells were washed once with phosphate buffered saline to eliminate unbound protein before cells were added. A hemocytometer was used to count the cells in endothelial basal medium. The cells were added to an immunolon 1^{TM} 96 well plate (Dynatech Laboratories Inc.). The final concentration of the cells was approximately 20 000 per well in 100 μl of endothelial basal medium. After cells were added to the appropriate wells, the plate was incubated for 2 h at 37 °C. Following incubation, the wells were washed 3 × with endothelial basal medium. The washed plates were assayed using the CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI, USA). Absorbance of light at 492 nm was measured using a Vmax kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). Cells were visually observed for spread morphology on the surface.

In vitro adhesion of ECs to Ti, SS and PS surfaces coated with fibronectin, vitronectin, fetal bovine serum, or peak 1 fibrinogen γAγA were compared to adhesion to these surfaces with only BSA or no coating. Anti-integrin antibodies and peptides were incubated with the cells 10 min prior to addition of the mixture to the 96 well plate. All samples were used in triplicate for each experiment. Each experiment was repeated at least once in order to verify the results. Sample sizes were not added between experiments due to the adsorbance variances between experiments. However, the relative comparisons between controls and experimentals remained the same between experimental runs. Therefore, results are reported as the mean of one experiment ($n = 8$) by cell numbers or mean percentages with standard errors or standard deviations as indicated. Standard student's t-tests were used to determine statistical significance at the $P < 0.05$ level.

2.4. Fibrinogens

Peak 1 fibrinogen γAγA, peak 1 fibrinogen Fr I-9 [32] (a fibrinogen fragment which lacks approximately 100 carboxyl-terminal residues from each Aα–chain, including the 572–574 RGD sequence, and is bivalent with respect to the γA–chain platelet binding sites), and fibrinogen fragment D1 [32], which contains a monovalent γA-chain without the Aα–chain 572–575 RGDS binding site were used in this study to try to identify the fibrinogen binding sites used by endothelial cells to support adhesion. These fibrinogens were prepared as previously reported [32].

2.5. Monoclonal antibodies

Monoclonal antibodies used were LM609, an anti-vitronectin receptor, $\alpha_v\beta_3$, (50 μg/ml) antibody [33], JB1a, an anti-β_1 antibody (1 : 100 dilution from Ascites fluid), CLB–706 (50 μg/ml), an anti-α_v antibody (Chemicon International Inc., Temecula, CA, USA), and 50 μg/ml of a control mouse IgG (Sigma). The anti-integrin antibodies were added to the cells 10 min prior to addition of the cells to the wells.

2.6. Peptides

Peptide inhibitors of fibrinogen binding to platelets were tested as inhibitors of endothelial cell adhesion to immobilized fibrinogen on metal and plastic surfaces. The peptide LGGAKQAGDV (L10), a γA-chain carboxyl terminal fibrinogen mimetic, GRGDSP (RGD), an RGDS containing peptide, the control peptide GRGESP (RGE) and a control scrambled version of GRGDSP, PGRSGD (PGR) were tested in the adhesion assay at approximately 1.5 mM concentration. Amino acid quantity and sequences of the peptides were verified by St Jude Children's Research Hospital Biotechnology Center Laboratories (Memphis, TN). The methods used for the synthesis, purification and characterization of these peptides have been described previously [34].

2.7. Protein assay

A 0.01% Coomassie Brilliant Blue G250 (Bio-Rad Laboratories, Richmond, CA) solution was utilized to measure the amount of albumin adsorbed to the surfaces after washing. BSA (0.5%) was added to six wells containing Ti discs, six wells containing SS discs and six

PS wells and allowed to incubate at room temperature for one hour. The wells were then washed with phosphate buffered saline twice (100 µl/wash). The Coomassie blue was added to the coated surfaces and allowed to incubate at room temperature for 10 min. The color was measured at a wavelength of 595 nm. Surfaces with no albumin ($n = 6$ for each surface) were used as controls. A standard curve for BSA from 0.1 µg to 50 µg was used to estimate the amount of albumin on the surface after the wash. All experimental points were within the linear part of the standard curve.

3. Results

Adhesion results demonstrated that fibronectin coated surfaces supported adhesion of more ECs (on all three surfaces) than the other proteins used in this study (Fig. 1). EC adhesion was the most extensive in the PS wells coated with fibronectin. In contrast, PS wells coated with serum and PS control wells (control wells for each metal and PS were only postcoated with BSA with no overnight coating) supported very little or no adhesion of ECs. Therefore the adhesion with other coatings was expressed as a percentage of that for fibronectin-coated PS wells (Fig. 1).

The bare metal surfaces (non-coated) supported more EC adhesion than the non-coated PS wells (Fig. 1). After coating with BSA, adhesion to the PS and the SS discs was blocked. The BSA-coated SS only supported 25% of the adhesion seen with non-coated SS (Fig. 1). However, the Ti coated with BSA supported almost as much (84%) EC adhesion as no coating (Fig. 1). This surprising result indicated that the BSA either did not adsorb to the Ti surface and was washed away or that the molecule adsorbed in such a way that it did not block the receptor mediated adhesion to the bare metal surface or that adsorption changed the conformation of the BSA such that it supported EC adhesion.

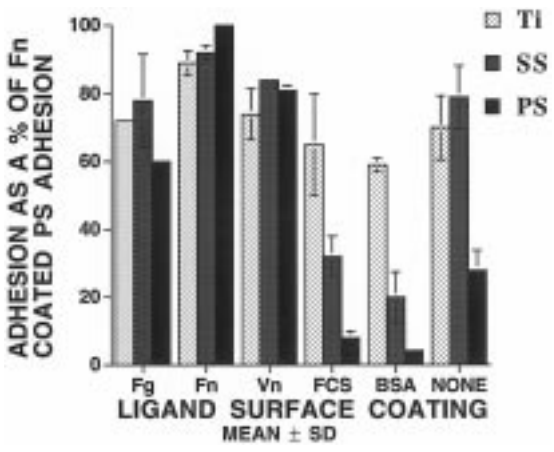

Figure 1 Adhesion of human microvascular endothelial cells to protein coated metal surfaces and polystyrene. Bars represent the adhesion as a percentage of fibronectin coated polystyrene adhesion after two hours incubation. Ti = titanium alloy, SS = stainless steel, PS = polystyrene, Fg = peak 1 fibrinogen γAγA, Fn = fibronectin, Vn = vitronectin, FCS = fetal calf serum, BSA = bovine serum albumin (control), none = noncoated or bare discs. Data presented as the mean ± standard deviation. $n = 3$.

In an attempt to distinquish between these alternatives, the washed metal surfaces were evaluated for the presence of BSA. Coomassie blue staining qualitatively revealed that as much, if not more, BSA remained on the Ti surface and on the SS as on the PS surface (Fig. 2). Therefore, the amount of BSA adsorbed to the Ti and SS surfaces was not the basis of the difference of cell adhesion to these metals. Next, the effect of increasing concentration of BSA from 0.01% to 5% used to post-coat the wells was examined. An increase in the BSA concentration resulted in a minor decrease in the number of cells that adhered to both metal surfaces (data not shown). In summary, the BSA did adsorb to the Ti, it only slightly suppressed cell adhesion to the protein coated Ti discs.

ECs adhered less extensively to fibrinogen than to coated surfaces (Fig. 1). Metal surfaces coated with fibrinogen supported the same amount of EC adhesion as no coating (however, these fibrinogen coated surfaces were post-coated with BSA). The PS and SS surfaces coated with fibrinogen supported more EC adhesion than the same surfaces only coated with BSA. This provides evidence that fibrinogen supports EC adhesion on PS and SS surfaces. Ti surfaces coated with fibrinogen γAγA, Fr I-9 or fragment D1 supported EC adhesion equally well. However, this adhesion was similar to that of serum coated Ti (data not shown). In this regard, PS coated with fibrinogen γAγA, Fr I-9 or fragment D1 supported more EC adhesion than the serum coated surface. Therefore, it is not clear that fibrinogen supported EC adhesion to Ti from these experiments.

The anti-$\alpha_v\beta_3$ antibody, LM609, partially blocked adhesion of ECs to fibrinogen, fibrinogen fragments (data not shown), fetal calf serum, or control (BSA only) coated Ti (Fig. 3). The anti–α_v antibody, CLB–706, partially inhibited adhesion on Ti coated with fibronectin, fibrinogen, serum and control surfaces. This

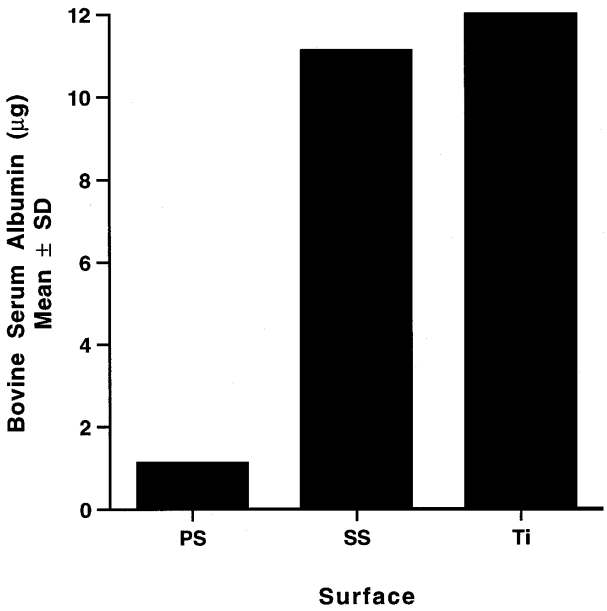

Figure 2 Albumin concentration (µg/ml) on the three surfaces after washing. Bars represent the albumin adsorbed to the surfaces after washing with phosphate buffered saline. Ti = titanium alloy, SS = stainless steel, PS = polystyrene. Data presented as the mean ± standard deviation. $n = 6$.

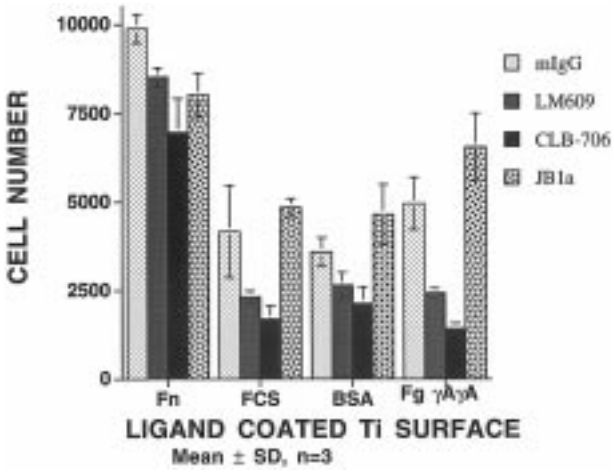

Figure 3 Endothelial cell adhesion to coated titanium in the presence of antibodies against integrins and integrin subunits. Fn = fibronectin, FCS = fetal calf serum, BSA = bovine serum albumin (control), Fg γAγA = peak 1 fibrinogen (γAγA), mIgG = control mouse IgG, LM609 is a monoclonal antibody which binds $\alpha_v\beta_3$, CLB-706 is a monoclonal antibody which binds the α_v integrin subunit, and JB1a is a monoclonal antibody which binds the β1 integrin subunit. Data presented as the mean cell number ± standard deviation. $n = 3$.

provides evidence of a role for the $\alpha_v\beta_3$ receptor in EC adhesion to coated Ti. The anti-β_1 antibody, JB1a, increased adhesion of ECs to fibrinogen and BSA coated Ti.

Finally, the peptide GRGDSP greatly inhibited adhesion of ECs to Ti coated with fibrinogen, its fragments or vitronectin when compared to the scrambled version of the peptide (PGRSGD) (Fig. 4). The control peptide GRGESP inhibited adhesion to the surfaces tested only to a minimal degree. The platelet binding site fibrinogen γA-chain carboxyl terminal mimetic peptide L10 partially inhibited adhesion of ECs to both fibrinogen coated and fibrinogen fragment coated Ti but not to vitronectin coated Ti. These data indicate that integrins which recognize the peptides GRGDSP and to some extent L10 play an important role in EC adhesion to protein coated Ti.

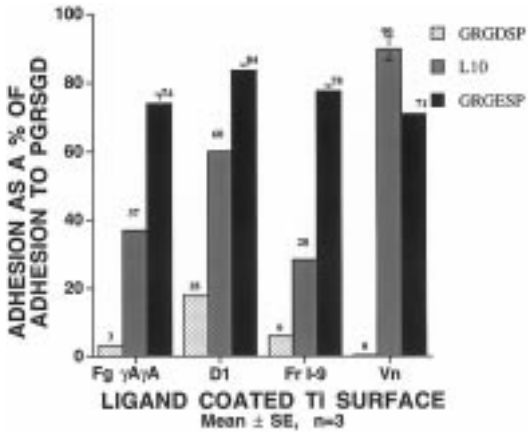

Figure 4 Endothelial cell adhesion to coated titanium discs after incubation with peptides. Fg γAγA = peak 1 fibrinogen (γAγA), Fr I–9 = peak 1 fibrinogen I-9, which lacks the Aα-chain 572–574 RGD sequences, D1 = a monovalent fragment of fibrinogen which lacks the Aα-chain 572–574 RGD sequence, but retains the γA-chain platelet binding region, Vn = vitronectin, PGRSGD = scrambled control version of peptide GRGDSP, L10 = LGGAKQAGDV. Data presented is adhesion as a mean percentage of adhesion to PGRSGD control peptide ± standard deviation.

4. Discussion

Surprisingly, the uncoated metal surfaces supported more EC adhesion than the uncoated PS surface. Also, BSA blocked adhesion to SS and PS surfaces, but did not block adhesion to Ti.

BSA is the most abundant protein in serum (4.5 grams/dl) and its ability to block cell adhesion to PS and to SS, but not to Ti, may be of importance in orthopaedics. The protein assays revealed that more BSA remained on the metal containing wells after washing than remained on the PS surfaces. However, the BSA left on the PS surface effectively blocked adhesion of ECs to this surface. Therefore, the BSA on the Ti surface was conformationally changed such that either it was recognized by specific cell receptors or it was no longer effective in blocking EC binding. However, increasing the concentration of BSA on Ti discs did not increase adhesion of ECs nor were amounts in excess of 1% more effective in blocking EC adhesion to Ti. Therefore, we conclude that ECs are not adhering to a conformationally changed BSA, but rather the BSA is no longer effective in blocking the adhesion. These data may provide a basis for understanding *in vivo* observations that tissues attach to Ti implants more securely than to SS. In this regard, Howlett *et al.* have shown that BSA adsorbs to Ti differently than to SS surfaces [35].

Control surface adhesion (BSA post-coating with no other adsorbed proteins) profiles were very similar to the profile of the surfaces coated with serum components. This may be due to the high concentration of BSA in serum, which may compete with the other proteins for adsorption to the surface and may be the predominant protein on the surface.

These assays demonstrated adhesion of ECs to fibrinogen γAγA, Fr I-9, D1, fibronectin, vitronectin, and fetal calf serum coated on metal discs (data not shown) as well as non-coated metal discs. SS and PS coated with fibrinogen (and post-coated with BSA) supported more EC adhesion than the same control surfaces. However, because BSA coated control discs of Ti supported EC adhesion to approximately the same extent as Fg treated discs, it is not possible to know from those data if ECs can adhere to the fibrinogen on the fibrinogen coated Ti. Using fibrinogen or its fragments as the ligand on the Ti surface, there is no increase in adhesion compared to albumin or serum coated discs. Furthermore, an ELISA assay using a specific monoclonal antibody (4A5, which binds the carboxyterminal end of the fibrinogen gamma chain [36]) indicated that fibrinogen was present after washing on all three surfaces (data not shown). However, the adhesion of ECs to fibrinogen coated Ti is also the same as the noncoated Ti controls. Therefore, there is no evidence that fibrinogen bound to Ti surfaces can support EC adhesion.

The $\alpha_v\beta_3$ integrin appears to have a role in adhesion of ECs to metal discs coated with serum components, albumin or fibrinogen as indicated by the antibody data. These results are consistent with the observations of others that $\alpha_v\beta_3$ is important in EC adhesion to immobilized proteins [33, 37]. The involved integrins recognize the GRGDSP peptide and to some extent the L10 peptide. The data presented here do not exclude the

role of other integrins in the adhesion of ECs to these protein coated surfaces. Further work is required to resolve this issue. The antibody and peptide results for the metal surfaces are very similar to that of the PS surface (data not shown). This indicates that similar receptors may be involved for adhesion of ECs to the different surfaces coated with the same proteins.

It has been demonstrated that integrins can be activated by the binding of certain monoclonal antibodies [38, 39]. Antibodies may activate receptors in two ways, either by crosslinking the receptors thus causing activation [40–43] or by activating receptors in a monovalent fashion by directly binding to the receptor [44, 45]. Examination of whether the anti-β_1 antibody activates integrins or increases adhesion through some other mechanism or whether the JB1a antibody (which is from ascites fluid) contained some factor which could have influenced cell adhesion was not addressed in this study.

5. Conclusions

Although the data presented here do not provide a basis for differential cell adhesion to surfaces, hopefully it will lay the groundwork needed for further studies and lead to the identification and use of a coated metal surface which will facilitate initial EC adhesion and therefore enhance capillary formation. Such a coated surface could provide the advantage of decreased healing time leading to earlier and possibly more bone formation about the implant to provide mechanical stability which is especially important in joint replacements. Earlier migration and more EC adhesion around the implant may provide another benefit by way of a decrease in exposure time of the implant to inflammatory cells such as macrophages which have been implicated as the mediators of osteolysis, and thus implant failure [46].

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