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PLATELET-ENDOTHELIAL CELL INTERACTIONS FOLLOWING
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ENVIRONMENTAL MEDICINE NATICK MA L R TRUSAL ET AL.

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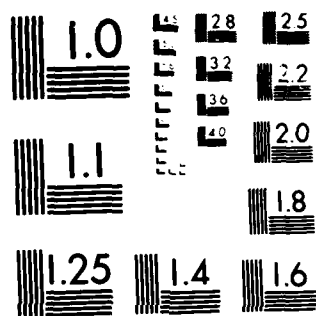
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platelets from WB demonstrated aggregation and extensive pseudopodia formation. Treatment of cells with Triton-X detergent resulted in exposure of a intracellular cytoskeleton. Most platelets attached to the cytoskeleton were located near the cell border and had one or more pseudopodia either in contact with extracellular material or penetrating the cytoskeleton. Adhesion of platelets to ECM may represent platelet-collagen or platelet-fibronectin interactions since both are produced by and incorporated into the ECM. Platelet interaction with endothelial cytoskeletons probably represented pseudopodia contact with the now exposed ECM located beneath the endothelial cells. The possibility that platelets also adhered to intracellular components could not be eliminated. These findings are in agreement with data from an isolated aorta freeze-thaw injury model. In addition, they tend to indicate that physical insult was not sufficient to induce platelet interaction with the endothelial surface, but that chemical modification (TX treatment) enhanced platelet deposition.

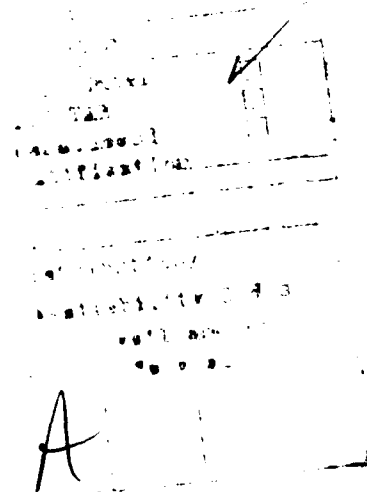
Title: Platelet-Endothelial Cell Interactions Following Freeze-Thaw
Injury or Detergent Treatment to Cultured Cells In Vitro

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ABSTRACT:

✓ Platelet interactions with cultured bovine endothelial cells were studied following freeze-thaw damage or detergent treatment. Platelets from whole blood (WB), platelet rich plasma (PRP) or gel filtered (GFP) did not interact directly with freeze-thaw damage endothelial cells. Freezing and thawing did result in the exposure of an extracellular matrix (ECM) located beneath the cells, which proved very thrombogenic. Platelets from all sources attached to both microfilament and amorphous components of the ECM, although only platelets from WB demonstrated aggregation and extensive pseudopodia formation. Treatment of cells with Triton-X detergent resulted in exposure of a intracellular cytoskeleton. Most platelets attached to the cytoskeleton were located near the cell border and had one or more pseudopodia either in contact with extracellular material or penetrating the cytoskeleton. Adhesion of platelets to ECM may represent platelet-collagen or platelet-fibronectin interactions since both are produced by and incorporated into the ECM. Platelet interaction with endothelial cytoskeletons probably represented pseudopodia contact with the now exposed ECM located beneath the endothelial cells. The possibility that platelets also adhered to intracellular components could not be eliminated. These findings are in agreement with data from an isolated aorta freeze-thaw injury model. In addition, they tend to indicate that physical insult was not sufficient to induce platelet interaction with the endothelial surface, but that chemical modification (TX treatment) enhanced platelet deposition.

Key Words: Endothelial cell, platelets, extracellular matrix, Triton-X.

INTRODUCTION:

The role of platelets in hemostasis is well documented (17,21). It is accepted that platelets do not interact with undamaged endothelium in vivo (2,22,31,37), with blood vessel segments in vitro (3,35,40) or when endothelium is removed from the intimal lining and cultured in vitro (6,14,44,48). Although prostaglandin I_2 (PGI_2) has been implicated in thromboresistance of intact endothelium (9,32) other reports lend doubt to this conclusion (12,13).

In contrast, when endothelium is damaged by various means some reports indicate that direct platelet-endothelial interactions occur (11,23,30) while others do not find such interactions (1,10,42,43,44,46). We have reported that when bovine endothelial cells are freeze-thawed in aorta segments in vitro and perfused with either bovine platelet rich plasma (PRP) or gel filtered platelets (GFP) there is no direct or generalized platelet adhesion to damaged endothelium (40,41). Platelets did readily adhere to subendothelial components and were often found between gaps of adjacent endothelial cells or in simultaneous contact with subendothelium and the borders of endothelial cells. The present study describes the use of an in vitro perfusion system for the evaluation of platelet interactions with endothelial cells cultured on plastic substrates. The study was designed to compare the finding to those from our previous study of platelet-blood vessel wall interactions following freeze-thaw injury. In addition to physical stress to the endothelium, it was of interest to determine the effect of chemical modification (detergent treatment) of the endothelial surface on platelet-endothelial interactions.

MATERIALS AND METHODS:

Processing and Identification of Endothelial Cells

Bovine aorta endothelial cells (BAEC) were obtained by collagenase (1 mg/ml) perfusion of the luminal surface of thoracic aortas obtained from a local slaughter house, essentially as previously described (28). Cells ($5-6 \times 10^5$ cells/ml) were counted and seeded into Leighton tubes (Costar, Cambridge, MA) containing a plastic (polymethylpentene) coverslip. Growth media consisted of Medium 199 (GIBCO, Grand Island, N.Y.) supplemented with 20% fetal bovine serum, 2mm L-glutamine, 50 μ g/ml gentamicin and 2.5 μ g/ml fungizone. Cultures were then placed in a 37°C, 5% CO₂ incubator until cell growth reached confluency in 2-3 days. Cultures treated with Triton-X (TX) were usually 6-7 days old.

Identification of endothelial cells was carried out by both transmission electron microscopy and immunofluorescence techniques, using the presence of Weibel-Palade (W-P) bodies (16,45) and Factor VIII antigen (25) as definitive markers, respectively.

Blood Processing

Whole blood was collected from jugular vein of unanesthetized calves into plastic centrifuge bottles containing anticoagulant (acid citrate dextrose). Platelet rich plasma (PRP) was prepared by centrifugation at 1500 rpm for 12-14 min at 22°C. Platelet poor plasma (PPP) was collected by centrifugation of PRP at 3300 rpm for 30 min.

Aggregometry

Both PRP and GFP were tested for their ability to aggregate in the presence of adenosine diphosphate (ADP) and collagen. These tests were carried out using a Payton aggregometer (Payton Assoc., Buffalo, NY) according to

established procedures (8). The final concentrations of ADP and collagen were 0.1 mg/ml and 0.26 mg/ml, respectively. Platelet counts were adjusted to $250,000 \text{ mm}^3$ before testing. All tests were carried out at 37°C with a stirring rate of 900 rpm. No platelet suspensions were used unless they demonstrated at least a 50% maximal aggregation response to both agents.

Gel filtration of platelets

Gel filtered platelets (GFP) were prepared by modification of previously published procedures (27,38). Basically, the procedure consisted of layering PRP onto a Sepharose 2B gel (Pharmacia Fine Chemicals, Piscataway, NJ) column conditioned with a complete Tyrodes buffer containing dextrose (1%) and albumin (3%). GFP were eluted into this same buffer, counted and tested for their ability to aggregate prior to perfusion.

Freeze-Thaw System

Leighton tubes, in which endothelial cells had been grown, were submerged in a 95% ethanol refrigerated water bath (Neslab, Portsmouth, NH). The rate of freezing (approximately $1^\circ\text{C}/\text{min}$ was measured by a thermocouple inserted in the Leighton tube. The freezing rate was recorded on a Leeds and Northrup (North Wales, PA) chart recorder. The Leighton tube media temperature was lowered to -15 or -20°C and then thawed in a 37°C water bath, while control tubes were maintained at 37°C .

Detergent Treatment

Coverslip endothelial cell cultures were first rinsed in phosphate buffered saline (PBS) and then immersed in a 0.5% solution of in PBS for 15-30 min (22°C) with occasional agitation. This was followed by multiple rinses in PBS to remove residual detergent before placing coverslips in the perfusion chamber.

Coverslip Perfusion System

Coverslips containing either control (37°C) or experimental (-15°C, -20°C, TX treated) endothelial cell cultures were removed from their respective Leighton tubes. After the handle portion was removed, one experimental and one control coverslip were placed in specially constructed siliconized glass perfusion tubes so that the cell monolayer faced the lumen of the tube (Fig. 3). The ends of the tubes were stoppered and attached to the perfusion system as detailed in Figure 1. Plastic pipettes, silicon stoppers, silastic tubing and siliconized flasks and perfusion chambers were used to prevent platelet activation. Platelet counts (PRP and GFP) were adjusted to 250,000 mm³ using, PPP or Tyrodes buffer, respectively. Platelet suspensions or whole citrated blood (WB) were perfused at a flow rate of 5 ml/min at 37°C, for 30 min. Following perfusion the system was allowed to drain, followed by flushing with PBS (15 min), and flow fixation for 30 min (5 ml/min).

TEM/SEM Processing

The plastic (polymethylpentene) coverslips used for SEM and TEM were processed as previously described (39). Briefly, coverslips were rinsed in PBS fixed in 2.5% buffered glutaraldehyde, post-fixed in 1% buffered osmium tetroxide and dehydrated in a graded series of ethanol. One portion of the coverslip was excised, critical point dried, mounted on stubs, sputter coated (Au/Pd) and examined by SEM. The remaining piece of coverslip for TEM was embedded in Epon-Araldite, polymerized, sectioned and stained for viewing.

RESULTS

Endothelial Cell Identification

Endothelial cell cultures exhibited positive cytoplasmic fluorescence after staining for the presence of Factor VIII antigen by indirect immunofluorescence

(Fig. 2). Only background fluorescence was noted in control cultures incubated with PBS or FITC. In addition, TEM demonstrated the presence of WP bodies in these cultured cells (Fig. 3).

Platelet Aggregation

Both sources of platelets (PRP and GFP) aggregated in the presence of ADP or collagen. Maximal platelet aggregation occurred in 5-10 minutes in response to ADP with GFP reaching a slightly higher maximum % aggregation. In contrast, platelets from PRP responded better to collagen. Both PRP and GFP reached maximum aggregation after collagen stimulation in 10-30 minutes. Attesting to the stability of bovine platelets, both PRP and GFP maintained 95% of their ability to aggregate in response to ADP or collagen, after storage for 12 hrs at 22°C.

In Vitro Perfusion

Control endothelial cell cultures perfused in vitro with PRP had no platelets attached to either endothelial cells or to the plastic coverslip (Fig. 4). This was also true of GFP perfused cultures. Freezing and thawing of the endothelial cell monolayers in detachment of many from the coverslip. Figure 5A illustrates a freeze-thaw (-20°C) damaged endothelial cell on a coverslip perfused with PRP. A similar culture perfused with GFP is illustrated in Figure 5B. These figures demonstrated the severe damage induced by freeze-thaw insult to damage included perforated plasma membranes and ruffled cell borders, as seen by SEM (Figs. 5A, B). Note, that although these cultures were perfused with both PRP and GFP, only one platelet was found to be in direct contact with the remaining cell body of an endothelial cell (Fig. 5A arrow). In general, platelets were found to be attached to the microfilament network exposed by the freeze-thaw procedure.

The extracellular matrix (ECM), exposed by the freeze-thaw procedure consisted of microfilaments of various diameters and amorphous material. Figure 6A is an SEM micrograph of this ECM network, while a more detailed structure can be seen in the TEM micrograph (Fig. 6B). Perfusion of WB, PRP or GFP across this ECM resulted in adhesion of platelets to the microfilament network. Figure 7A demonstrates the attachment of PRP to the ECM remaining on the coverslip following freeze-thaw. Note that most platelets are still disc shaped with few pseudopodia. In contrast, the platelets from WB demonstrated an aggregation response that included extensive shape changes and formation of many pseudopodia (Fig. 7B).

Figure 8A is a low magnification SEM showing the attachment of platelets (WB) to a TX extracted BAEC monolayer. Note that the vast majority of attached platelets are found between adjacent cells attached to filamentous strands on the substrate. In a higher magnification photomicrograph, (Fig. 8B) platelets (WB) were found interacting directly with microfilaments located between adjacent cytoskeletons (Fig. 8B). Some platelets remain disc shaped with short pseudopodia while others had morphological changes associated with long pseudopods contacting the extracellular filaments. Detergent-treated monolayers perfused with PRP or GFP demonstrated similar adhesion results but the percent of activated platelets (pseudopodia formation) was decreased. Figure 9 illustrates a phenomena not seen in either control (37°C) or freeze-thaw (-15, -20°C) damaged monolayers; the presence of attached platelets on the surface of TX treated BAEC. Platelets (PRP) can be seen in direct contact with the remaining cytoskeleton and are especially associated with the cell border (Fig. 9). A similar finding was noted with GFP, as seen in Figure 10A. Many platelets are in contact with the remaining cytoskeleton. Most cytoskeletal-associated platelets had morphological changes associated with long pseudopodia.

In addition all were in the vicinity of the cell border. A higher magnification (Figs. 10B, 10C) indicated that platelet pseudopodia may have penetrated the cytoskeleton filamentous network.

TEM analysis of platelets bound to ECM material are illustrated in Figures 11 and 12. In Figure 11 a platelet (WB) still containing its dense granules is seen attached to microfilaments (100-120Å) and amorphous material of what appears to be ECM. A platelet (PRP) in contact with only amorphous material is illustrated in Figure 12. Note the absence of pseudopodia and the presence of a dense body and microtubules in the attached platelet. No apparent filaments of any size are seen in contact with the platelet.

DISCUSSION

Since Factor VIII is not found in either smooth muscle cells or fibroblasts (25), but is specific to endothelial (36), it was concluded that cultures obtained from bovine aortas were composed of endothelial cells. This was confirmed by the demonstration of WP bodies in these BAEC cultures (16,45).

In the recent work demonstrating the ability of BAEC to produce a subendothelial matrix, cells were grown on glass coverslips and exposed only to washed platelets (7). The present study utilized plastic (polymethylpentene) substrates which removed the possibility of glass induced aggregation of platelets and included different platelet sources such as PRP and WB.

Platelet perfusions past control (37°C) BAEC cultures were similar in outcome to the results obtained using perfused aorta segments in vitro (40,41). In both in vitro systems, platelets from PRP, GFP or WB did not interact with undamaged endothelial cell monolayers.

After freeze-thaw insult of BAEC cultures, generalized adhesion of platelets from any source to damaged cells was not demonstrable. This is in agreement with freeze-thaw damage to in vitro perfused aorta segments (40,41)

and endothelial injury induced by heat (44) or hypotonic treatment (6). In contrast to these findings cultured endothelial cells treated with thrombin (14) or 2-chloroacetaldehyde (48) permits platelet-endothelial interactions following such treatment. These studies suggest that physical insults to the endothelial cell surface are insufficient to cause platelet interactions but that chemical modifications may allow platelet deposition.

It was also our intent to minimize the influence of subendothelial components on platelet adhesion and aggregation, by culturing BAEC in vitro. However, freeze-thaw insults to one week old cultures revealed that BAEC produced an ECM beneath the cells. It was therefore possible that this matrix was substantially like in vivo subendothelium and capable of eliciting a thrombogenic response.

In this regard, our results clearly demonstrated the thrombogenicity of this endothelial cell produced matrix. Platelets derived from PRP, GFP or WB readily adhered to the filaments of the matrix (Fig. 7A). Only platelets in WB demonstrated an ability to aggregate as evidenced in Figure 7B. This is not surprising since ADP may be released from red blood cells found only in the WB perfusate and not present in either PRP or GFP suspensions. Degranulation of platelets attached to the ECM was not noted and in only WB cultures was extensive pseudopodia formation found. This difference from earlier studies (F) may be related to glass versus plastic substrates.

It has been shown that BAEC produce both collagen (19,24,26) and fibronectin (5,19,29) and incorporate them into an ECM. The ability of fibronectin to bind collagen (4,15,33) and promote cellular adhesion, when attached to substrates has also been studied (19). It is also interesting that fibronectin is found on platelet membranes (4) and alpha granules (49) and as such may be a receptor for platelet-collagen interactions (4). In addition,

investigators using bovine corneal endothelial cells demonstrated that the apical cell surface was devoid of fibronectin and non-thrombogenic, whereas the basal cell surface contained fibronectin and was thrombogenic (18). When the apical cell surface was mutagenized with 2-choloracetaldehyde, fibronectin was found on both the upper and lower cell surface (48). These altered cells now possess the ability to bind platelets. The binding of platelets to ECM filaments exposed following freeze-thaw may therefore be the result of platelet-collagen fibronectin interactions.

Since platelets readily interacted with the extracellular filaments of the ECM, it was of interest to determine whether platelets would also interact with the intracellular cytoskeleton following exposure by TX treatment. Such treatment digests the plasma membrane leaving only the nucleus and cytoskeleton attached to the substrate (34,47). It became evident that detergent-prepared cytoskeletons more readily interacted with platelets from all sources than before treatment. Most attached platelets were usually located near the cell periphery (Figs. 9A, 10A) and often had pseudopodia projecting through the cytoskeleton (Figs. 10B, 10C) or onto the plastic substrate (Fig. 10A).

In these TX-treated cultures, the presence of platelets in direct contact with cytoskeleton components may be interpreted in several ways. Perhaps platelet interaction with the cytoskeleton of BAEC in reality represented pseudopodia contact with the now exposed ECM components located beneath the intracellular cytoskeleton. The recent demonstration of intracellular fibronectin in the cisternae of endoplasmic reticulum and secretory vesicles of fibroblasts (20) raises another possibility. Penetration of the cytoskeleton by platelet pseudopodia may represent platelet response to intracellular sites of fibronectin. It is also possible that intracellular filaments not previously shown to elicit platelet adhesion or activation may be responsible for mediating such a response.

Although TEM micrographs clearly demonstrated platelets in direct contact with both microfilaments and amorphous material, it was not possible to definitely state the material was extracellular in origin. Therefore, additional studies are needed to elucidate the response of platelets to both specific intracellular and extracellular endothelial components, as they may relate to in vivo subendothelial structures.

In summary, freeze-thaw damage of BAEC cultures induced platelet interactions which were quite similar to those noted when studying platelet-vessel wall interactions following freeze-thaw insult of isolated aortas. It may therefore be possible to use this in vitro endothelial culture model to study the effects of freeze-thaw insult of endothelial cells derived from those vascular beds (peripheral) most affected by frostbite. Such studies may further define the role of platelet-vessel wall interactions in the hemostasis induced tissue injury associated with this form of cold trauma. In addition to freeze-thaw injury this in vitro model has been shown to be useful in the study of platelet interactions with cytoskeletal components and adds additional evidence that chemical treatment of the endothelial cell surface can result in modifications of the endothelium which enhances platelet deposition.

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DISCLAIMER:

The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other official documentation.

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FIGURES

- Figure 1 - Perfusion apparatus used to perfuse monolayer endothelial cell cultures with platelet suspensions.
- Figure 2 - Light micrograph of bovine endothelial cells exhibiting positive immunofluoresence for Factor VIII antigen, x 420.
- Figure 3 - Bovine endothelial cell containing Weibel Palade bodies. Note rod like structure (arrows), x 1,915.
- Figure 4 - SEM micrograph of control (37°C) bovine endothelial cells in monolayer culture perfused with PRP. Note platelets are not in contact with cells or plastic substrate, x 1,125.
- Figure 5A - SEM micrograph of freeze-thaw (-20°C) damaged endothelial cell. Nucleus is visible but cell membrane is perforated and cell border is ruffled. Note ECM attached to the substrate beneath the cell and several attached platelets (PRP), x 2,275.
- Figure 5B - SEM micrograph of the remaining nucleus of a freeze-thaw (-20°C) damaged endothelial cell. Note extensive ECM with adhering platelets (GFP), but not to remaining portion of endothelial cell, x 2,390.
- Figure 6A - SEM micrograph of ECM produced by bovine endothelial cells, x 7,635.

- Figure 6B - TEM micrograph of ECM. Note microfilaments (open arrows) and amorphous material (closed arrows), x 40,615.
- Figure 7A - SEM micrograph of PRP attached to ECM material. Note most platelets are still disc shaped with small or no pseudopodia, x 2,325.
- Figure 7B - SEM micrograph of platelets from WB attached to ECM. Note aggregation and extensive shape and pseudopodia changes in contrast to Fig. 7A, x 5,750.
- Figure 8A - Low power SEM micrograph of TX-treated endothelial cell culture perfused with WB. Note the majority of platelets are attached to ECM between the cells (arrows) or the region of the cell border, x 470.
- Figure 8B - Higher power SEM micrograph of platelets (WB) interaction with substrate filaments in TX-treated culture, x 5,285.
- Figure 9 - SEM micrograph of platelets (PRP) in direct contact with the cytoskeleton of a TX-treated endothelial cell. Most are still disc shaped but some have short pseudopodia (open arrow). Note what is probably ECM on the plastic substrate (closed arrows). x 5,180.

Figure 10A - SEM micrograph of platelets (GFP) on surface of TX-treated endothelial cell. Note attached platelets are near the cell border and have long pseudopodia (arrows). See inserts for higher magnification, x 5,250.

Figure 10B - Higher magnification view of platelets (GFP) on edge of cellular cytoskeleton, x 14,000.

Figure 10C - Higher magnification view of platelet (GFP) pseudopodia (arrow) penetrating cellular cytoskeleton of TX-treated cell, x 11,000.

Figure 11 - TEM micrograph of platelet (WB) in contact with microfilaments of ECM, x 23,920.

Figure 12 - TEM micrograph of platelet (PRP) in contact with amorphous material of ECM, x 62,500.