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Role of the vascular endothelium

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The intact vascular endothelial surface is considered to be 'non-thrombogenic', and blood platelets usually fail to adhere to it. In this role, the endothelium serves to maintain the integrity of the vascular system by preventing the escape of blood and by preventing the build-up of solid thrombus within the vessel, which would compromise blood flow. A possible explanation for the non-thrombogenic effect of the endothelium is the presence of prostacyclin (PGI_2), the potent inhibitor of platelet aggregation and adherence, which is produced and released by the endothelium in response to various stimuli. Removal of PGI_2 from the endothelium by four different methods did not increase baseline platelet adherence, but did increase thrombin-induced platelet adherence from 4 to 60%. Addition of exogenous PGI_2, at low concentrations reversed the enhanced thrombin-induced platelet adherence under these conditions. Although it is unlikely that prostacyclin is the sole factor regulating platelet adherence to the endothelium, it appears to play a major role in the interaction of platelets with components of the blood vessel wall.

Introduction

A decade ago, it had been known for some time that the vascular endothelium functioned differently from other cells, but the remarkable capacity for the endothelial surface to maintain non-thrombogenic properties in the face of myriads of procoagulant substances and activated platelets, though appreciated, was poorly understood. During the previous two decades, many advances were recorded in our understanding of the ultrastructural features of endothelium, responses to vascular injury, exchange of fluid and nutrients across the endothelial layer and the migration of blood cells into the tissues (Majno 1965; Florey 1966). The past decade has witnessed a better understanding of endothelial cell biochemistry and physiology following the development of techniques that permitted these cells to be grown in tissue culture.

Normal functions of the endothelium

In general, three functions have been delineated for the vascular endothelium. It supplies small molecular nutrients to the subendothelial structures, acts as a barrier to large macromolecular substances, and presents a non-thrombogenic surface to circulating blood constituents. The endothelium forms a continuous monolayer, 0.5–1.2 μm in thickness. The long axis of the individual cells constituting this monolayer parallels the direction of blood flow. Each endothelial cell is sheet-like in character with an extremely high surface:volume ratio.

The luminal surface of the endothelium is covered by an ultrathin micropolysaccharide coat referred to as the glycocalyx. The cell's plasma membrane is a trilaminar structure similar to

other biological membranes. Intercellular junctions are of three main varieties: (1) tight junctions are very localized areas of fusion of the two outer plasmalemmal leaflets of adjacent cells and are local permeability barriers to protein molecules; (2) gap junctions are areas of close cellular apposition that are not fused and are important in the intercellular transfer of ions; (3) the major portion of intercellular contact is an overlapping of peripheral cytoplasmic portions of neighbouring cells with an intercellular space of 15–20 nm.

Several different types of membrane-related structures are likely to be important in cellular transport. Vesicles known as caveolae intracellulares, measuring about 70 nm in diameter, are distributed along both luminal and basal plasma membranes. They are fused with the membrane to form flask-shaped structures; occasionally several may fuse to form a transcellular channel. Phosphatase activity has been demonstrated within these structures, but no relation with ADP-induced platelet aggregation has yet been reported. A second type of vesicle referred to as a 'pit' can be seen, which measures 80–120 nm in diameter. These pits function in the selective uptake of substances, particularly proteins, and include sites of enzyme activity and lipoprotein receptors.

Non-thrombogenic features

The emphasis in this paper is on those properties of the vascular endothelium that enable it to maintain its normal non-thrombogenic surface. Possible factors and mechanisms that may be involved are listed in table 1.

Table 1. Factors in endothelial non-thrombogenicity

electrostatic repulsion
surface ADPase
heparans–proteoglycans
plasminogen activator
thrombin binding
prostacyclin

Both the intact platelet and endothelial cell have negatively charged membranes at physiological pH and thus are mutually repelled by each other. It is believed that the surface of the endothelium is influenced by the presence of heparans and glycosoaminoglycans. Of particular interest has been the demonstration that the platelet contains an enzyme capable of liberating and degrading heparan sulphate associated with the surface of the endothelial cell (Buonassisi & Root 1975). Heparan sulphate is chemically related to heparin and at high concentrations shows anticoagulant activity. Thus, the platelet may be able, through an as yet unidentified stimulus, to modify directly an antithrombogenic property of the endothelial cell to enhance local haemostatic mechanisms.

An enzyme with ADPase activity has been found to be associated with the endothelial surface and would have an obvious role in preventing platelet aggregates from forming near intact endothelium (Heyns *et al.* 1974). The endothelium is known to produce an activator of plasminogen and carries the potential for activation of the fibrinolytic system to promote lysis of fibrin in clots or thrombi.

An interesting new role for the endothelium has been suggested by the recent work of Lollar & Owen (1980). These workers have demonstrated that the binding of thrombin to the endothelium is an important primary mechanism for the rapid removal of thrombin from the

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circulation and facilitates its subsequent association with anti-thrombin III (AT-III) to form a thrombin–AT-III complex.

Perhaps the most important non-thrombogenic property of the endothelium relates to its ability to produce and release prostacyclin. Moncada *et al.* (1976) have shown that arachidonate metabolic machinery exists in endothelial cells to convert arachidonic acid and cyclic endoperoxides to prostacyclin, a most potent inhibitor of platelet aggregation and adhesion.

Platelet adherence studies

To delineate more clearly the role of prostacyclin, we have performed studies on a platelet adherence system with cultured vascular cell monolayers (table 2). Prostacyclin was measured in these studies by using a radioimmunoassay for 6-keto-$PGF_{1\alpha}$, the stable end product of prostacyclin (Czervionke *et al.* 1978, 1979).

Table 2. Effect of bovine thrombin on adherence of platelets (percentages)

(Monolayers were rocked with 1 ml incubation medium (i.m.), with or without 0.5 U bovine thrombin, and 0.5 ml ^{51}Cr-labelled platelets for 30 min at 37 °C. Adherence was determined by the method of Czervionke *et al.* (1978). Values given are means ± s.e. for at least six dishes.) These data are taken from Fry *et al.* (1980) *Blood* **55**, 271.

	control (i.m.)	bovine thrombin, 0.5 U
endothelium		
venous	1.5 ± 0.2	4.0 ± 0.5
haemangioendothelioma	2.6 ± 0.7	66.9 ± 1.9
arterial smooth muscle	2.4 ± 1.0	79.4 ± 0.3
arterial fibroblasts	1.3 ± 0.4	79.4 ± 0.9
empty dish	2.1 ± 0.1	77.7 ± 2.7

To test the effect of prostacyclin in the platelet adherence system we used four different approaches to eliminate it from the endothelium.

Aspirin (ASA) is known to acetylate the cyclo-oxygenase of the platelet and to inhibit thromboxane A_2 formation. Therefore, we chose to treat the endothelial monolayer with aspirin so that in a similar way the endothelial cyclooxygenase would be inhibited and prostacyclin production would cease. We tested this possibility by using our assay for 6-keto-$PGF_{1\alpha}$ and the thrombin-induced platelet adherence system.

The results are shown in figure 1. In the absence of thrombin, there was no increased platelet adherence despite inhibition of prostacyclin formation. In the presence of thrombin and in the absence of aspirin, 6-keto-$PGF_{1\alpha}$ increased and there was little platelet adherence. When the endothelium was treated with 0.01 mM aspirin, thrombin still caused significant release of 6-keto-$PGF_{1\alpha}$, and no increase in platelet adherence occurred. However, treatment of the endothelium with 1 mM aspirin prevented the formation of 6-keto-$PGF_{1\alpha}$ even in the presence of thrombin, and platelet adherence increased to 44%.

In additional studies we demonstrated that the effect of aspirin on the endothelium was temporary (figure 2). When the aspirin-treated endothelium was removed from contact with the aspirin and restored to culture conditions 2 h later, thrombin caused significant 6-keto-$PGF_{1\alpha}$ release, and platelet adherence returned to normal. When cycloheximide, an inhibitor of

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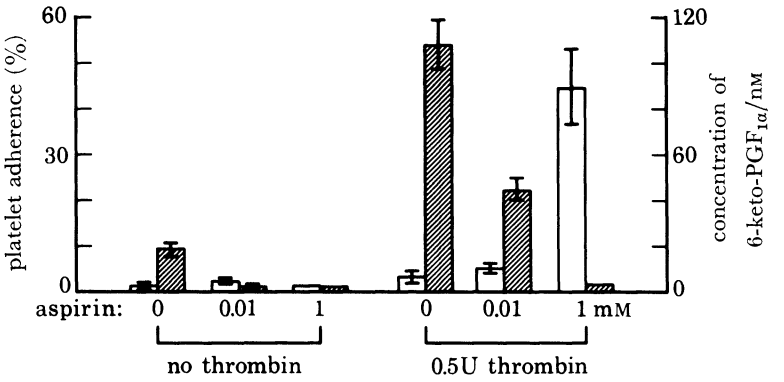

FIGURE 1. Platelet adherence (open columns) to untreated and aspirin (ASA)-treated endothelium compared with 6-keto-$PGF_{1\alpha}$ release (shaded columns). ASA or buffer control was incubated with the monolayer for 30 min at 37 °C, with rocking. The preincubation solution was removed and the dish was washed twice. Thrombin or buffer control was added, followed immediately by ^{51}Cr-labelled platelets (for adherence) or unlabelled platelets (for PGI_2 determinations). The monolayer was rocked for 30 min at 37 °C. The percentage adherence was calculated by dividing counts per minute of cells attached to the monolayer, multiplied by 100, by total counts per minute added to the dish. 6-Keto-$PGF_{1\alpha}$ released into the supernatant was determined by radioimmunoassay.

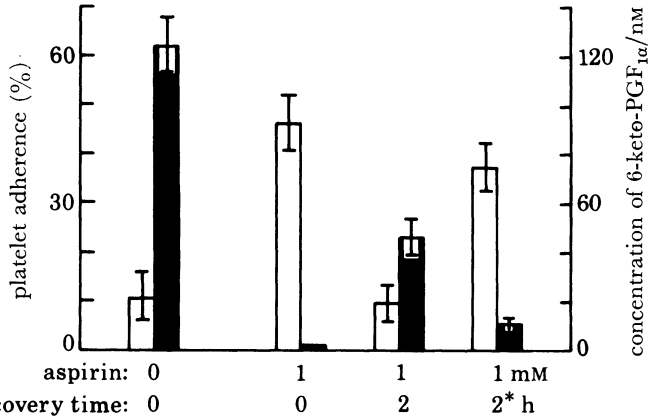

FIGURE 2. Duration of the aspirin effect on endothelium as reflected by 0.5 U thrombin-induced platelet adherence (open columns) and 6-keto-$PGF_{1\alpha}$ release (solid columns). ASA or buffer control was incubated with the monolayer for 30 min at 37 °C, with rocking. The preincubation solution was removed and the monolayer was washed twice. The designated monolayers were layered with 2 ml cultured medium with and without 2.5 μg/ml cycloheximide, incubated in a 5% CO_2 atmosphere at 37 °C for 2 h (recovery period), and rewashed twice. All monolayers were then treated with thrombin and platelets. *, With cycloheximide. From Czervionke *et al.* (1979) *J. clin. Invest.* **63**, 1089.

protein synthesis, was added to the endothelial culture during the recovery period, little 6-keto-$PGF_{1\alpha}$ was released, and platelet adherence remained abnormal.

Prostacyclin was also removed from the platelet adherence system by using an incubation system containing a rabbit antibody against 6-keto-$PGF_{1\alpha}$, which cross-reacts with prostacyclin. In the presence of the antibody, thrombin-induced platelet adherence to endothelium increased from 7.7% to 39.1%.

In the third set of experiments, the endothelium was subjected to repeated incubation with thrombin. As demonstrated by Czervionke *et al.* (1979), after the initial exposure to thrombin, the cultured endothelium was unable to respond to a second thrombin stimulus with a release

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of PGI_2. When platelet adherence studies were performed under these conditions in the absence of prostacyclin, platelet adherence was increased (see figure 3).

Using a culture of bovine pulmonary artery endothelium that did not produce prostacyclin, we performed additional platelet adherence studies (see table 3).

In the presence of thrombin, platelet adherence to the endothelial cells, which were unable to produce prostacyclin, was 78%. Pretreatment of the endothelium with aspirin did not increase the platelet adherence.

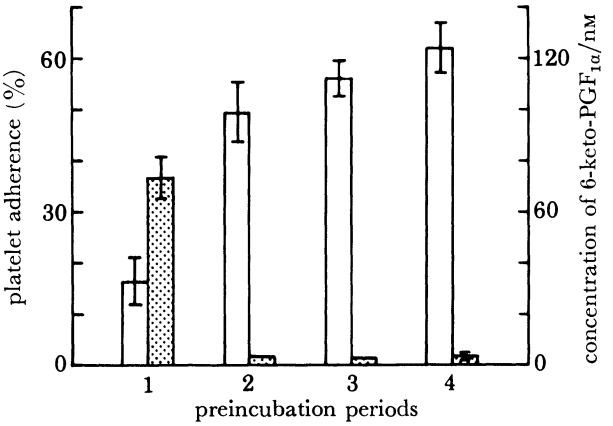

FIGURE 3. The 6-keto-$PGF_{1\alpha}$ release response with repeated exposure of the endothelial monolayer to thrombin. Five minutes after each addition of 0.5 U thrombin, the incubation mixture was completely removed. Fresh buffer containing thrombin was then added to the unwashed monolayer, which was reincubated, with rocking, for 5 min. This procedure was repeated 3 times. Platelet adherence (open columns) and 6-keto-$PGF_{1\alpha}$ concentrations (stippled columns) were determined. From Hoak *et al.* (1979) In *Florence International Meeting on Myocardial Infarction* (*Int. Cong. Ser.* no. 491), vol. 1, pp. 289–298. Excerpta Medica.

TABLE 3. RESPONSES OF BOVINE PULMONARY ARTERY ENDOTHELIUM, NO PGI_2 PRODUCED

(Platelet adherence was performed as in figure 1, except that the source of endothelium was a bovine pulmonary artery that failed to produce prostacyclin.)

	platelet adherence (%)
control	3
thrombin (0.5 U)	78
thrombin + 100 mM PGI_2	16
ASA (1 mM) + thrombin	69
ASA + thrombin + PGI_2	14

In each of the four sets of experiments used to remove prostacyclin from the endothelium, baseline platelet adherence did not increase. However, when thrombin was added to the incubation system, in every instance platelet adherence increased significantly and this increase was prevented by the addition of exogenous prostacyclin to the incubation media. Thus, prostacyclin appeared to be an important component to maintain platelet adherence at a low value when thrombin was present in the incubation system with the endothelium.

In order to study the effect of exogenous prostacyclin on platelet adherence to different types of vascular cells, the monolayers were pretreated with 1 mM ASA to block endogenous

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production of prostacyclin by thrombin. In order to determine whether some cell types were more sensitive to prostacyclin, concentrations from 25 to 150 nM were used. Fibroblasts were chosen as a representative cell type from the subendothelium, since values for smooth muscle and fibroblasts were not significantly different. Mouse fibroblasts were used as a control for the haemangioendothelioma. Figure 4 shows that platelet adherence to the venous endothelium, and haemangioendothelioma, decreased markedly with as little as 25 nM prostacyclin. In contrast, the platelet adherence to fibroblasts in the presence of thrombin was extremely resistant to the effect of prostacyclin.

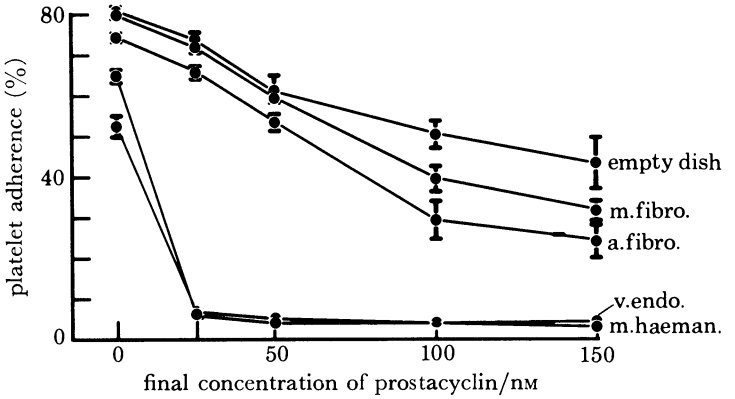

FIGURE 4. Inhibition by exogenous PGI_2 of 0.5 U thrombin-induced platelet adherence to 1 mM aspirin-treated cell layers. All monolayers were incubated for 30 min with 1 mM aspirin in incubation medium. After rinsing twice, platelet adherence was determined as described in the legend to figure 1. PGI_2 was added just before thrombin and platelets to achieve the concentrations shown. Abbreviations: a. fibro., arterial fibroblasts; v. endo., venous endothelium; m. haeman., mouse haemangioendothelioma; m. fibro., mouse L929 fibroblast. From Fry *et al.* (1980) *Blood* **55**, 271.

TABLE 4. EFFECT OF DAPA ON ADHERENCE OF THROMBIN-INDUCED PLATELET AGGREGATES

ASA (100 μM)	thrombin (0.067 U/ml)	DAPA (1.34 μM)	percentage adherence		
			endo.	fibro.	empty dish
.	×	.	38 ± 7	81 ± 4	86 ± 3
.	×	×	3 ± 4	71 ± 1	66 ± 1
×	×	.	68 ± 10	82 ± 2	85 ± 1
×	×	×	11 ± 6	67 ± 7	66 ± 3

In studies designed to test whether the effects of the thrombin were entirely on the platelets in the adherence system, we have obtained additional results suggesting that prostacyclin is not completely responsible for the non-thrombogenic character of the endothelium. DAPA (dansyl-arginine-*N*-(3-ethyl-1,5-pentanediyl) amide, an inhibitor of the active site of thrombin (Nesheim *et al.* 1979) was added to platelet aggregates that had formed when thrombin was added to ^{51}Cr-labelled platelets in an aggregometer. After the DAPA was mixed with the platelet aggregates, they were transferred to vascular cell monolayers to determine their adherence (see table 4).

DAPA prevented thrombin-induced platelet adherence to the endothelium, but failed to influence platelet adherence to fibroblasts or to the surface of the empty dish control. Even

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when ASA-treated endothelium was used, the presence of DAPA prevented significant adherence to the endothelium.

Our studies to date suggest a significant role for prostacyclin in the maintenance of a normally functioning endothelium. We have been concerned with control mechanisms for prostacyclin production and release.

Prostacyclin formation and release

Preincubation of cultured endothelial cells with 1 mM TMB-8 (Malagodi & Chiou 1974), an antagonist of intracellular calcium ions, or with 4 mM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic nucleotide phosphodiesterase activity, blocked prostacyclin release

Table 5. Effect of PGI_2 and 3-isobutyl-1-methylxanthine (IBMX) on the amount of cyclic AMP (picomoles) in endothelial cell monolayers

		amount of cAMP in 4.5×10^5 cells	
treatment	number of experiments	control	IBMX (4 mM)
control	7	2.16 ± 0.26	5.55 ± 0.57
PGI_2 (400 nM)	7	2.86 ± 0.46	11.81 ± 1.93

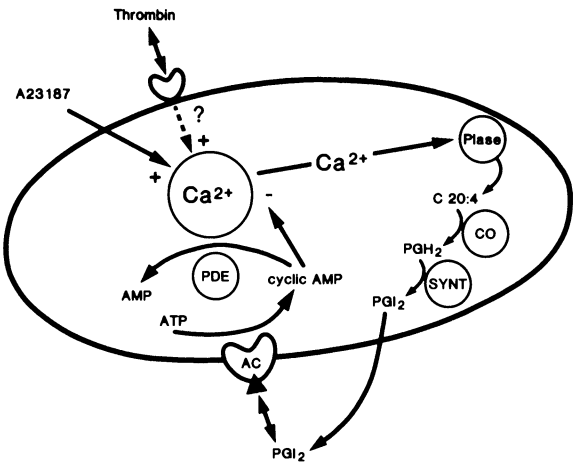

Figure 5. Hypothetical mechanisms involved in prostacyclin production and release. Abbreviations: AC, adenylate cyclase; CO, cyclo-oxygenase; PDE, phosphodiesterase; PGI_2, prostacyclin; Plase, phospholipase; SYNT, prostacyclin synthetase.

induced by thrombin or the calcium ionophore A23187, decreased arachidonic acid-induced release by about 50%, but had no effect on PGH_2-induced release (Brotherton & Hoak 1980). Radioimmunoassay of cyclic AMP in the endothelium showed that the basal level (2.16 ± 0.26 pmol of cyclic AMP per 4.5×10^5 cells) was increased by an average of 2.6-fold with 4 mM IBMX. As table 5 shows, prostacyclin (0.4 μM) had no significant effect on cyclic AMP levels in the absence of IBMX, but caused a twofold increase with 4 mM IBMX.

These findings suggest that an increase in the intracellular concentration of cyclic AMP antagonizes the effect of agents that require calcium ions for the induction of prostacyclin

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release. In addition, high cyclic AMP phosphodiesterase activity in the endothelium may protect against a negative feedback mechanism involving activation of adenylate cyclase by released prostacyclin. Figure 5 is a diagram of hypothetical reactions involved in prostacyclin control mechanisms.

Conclusion

The endothelium has a number of important functions. In this presentation, its normal role in platelet – vessel wall interactions has been emphasized. Prostacyclin appears to play a key role in preventing the aggregation of platelets and their adherence to the vascular wall cells when a thrombogenic stimulus such as thrombin is present.

The failure of high concentrations of prostacyclin to completely block thrombin-induced platelet adherence to smooth muscle cells and fibroblasts suggests that these cells either lack a component normally found in endothelium or produce a substance that promotes adherence. Possible differences include collagen production (type and amount) by smooth muscle and fibroblasts or, for endothelium, interactions at the surface that enhance the effect of prostacyclin. Monolayers derived from normal endothelium or from a malignant type of endothelium (haemangioendothelioma) exhibit some property in addition to prostacyclin that prevents thrombin-induced platelet adherence. Therefore, despite its ability to decrease adherence to all of the cell types tested, it is unlikely that prostacyclin is the sole factor regulating platelet adherence.

We are in an era in which there has been a rapid accumulation of new information about the endothelium and the interactions of platelets with the vessel wall. One can expect that the future will be equally challenging and rewarding. It is quite likely that discoveries of congenital and acquired defects of the endothelium will parallel those already described for platelet and coagulation factors. Hopefully, these advances will ultimately be reflected in the development of better preventive and therapeutic approaches to thrombotic disease.

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