Platelet-derived Interleukin 1 Induces Human Endothelial Adhesion Molecule Expression and Cytokine Production

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Summary

Interleukin 1 (IL-1) plays a central role in the regulation of the body's response to infectious and inflammatory stimuli. Recent evidence has shown that human platelets express a cell associated form of this proinflammatory cytokine very rapidly following activation. Since one of the earliest events in inflammation is frequently the rapid adhesion of platelets to injured endothelium, it was of interest to determine whether platelets express IL-1 in a functionally relevant form that can alter the phenotype of human endothelial cells in vitro. Thrombin activated platelets induced significant expression of the adhesion molecule intercellular adhesion molecule 1, as well as secretion of the IL-1 inducible cytokines IL-6 and granulocyte macrophage colony stimulating factor by cultured human umbilical cord and saphenous vein endothelial cells. This was inhibited by prior treatment of the platelets with antibody specific for IL-1. These results suggest that platelet delivered IL-1 might initiate and regulate some of the earliest phases of the inflammatory response. An additional observation of interest was differential induction of endothelial leucocyte adhesion molecule 1 by activated platelets on saphenous vein but not umbilical vein endothelial cells, which suggests functional heterogeneity of the endothelial cells.

The vascular endothelium normally maintains a thromboresistant luminal surface but following injury there is a rapid loss of this anticoagulant state, with consequent platelet binding and activation (1, 2). Endothelial cells also play an important role in the inflammatory response, where they undergo significant functional changes. These include increased expression of adhesion promoting molecules such as intercellular adhesion molecule-1 (ICAM-1)[1] and endothelial leucocyte adhesion molecule-1 (ELAM-1), thus leading to the binding and extravasation of leukocytes, as well as the release of soluble mediators (3, 4). Many of these changes can be mimicked in vitro by the addition of recombinant cytokines such as IL-1 and TNF to endothelial cell monolayers (5, 6). In addition local injection of IL-1 or TNF results in rapid recruitment of leukocytes from the blood (7, 8), and these cytokines can be detected at sites of inflammatory lesions, e.g., autoimmune sites and atherosclerotic lesions (9–11). These studies predict an important role for such cytokines in vivo in controlling the phenotype and function of endothelial and other inflammatory cells at sites of inflammation and immunological challenge. However the source and regulation of their production at the site of injury, especially in its earliest stages prior to leucocytic adherence and extravasation, has not been clearly identified.

One of us (C. M. Hawrylowicz) has recently demonstrated that following activation, human platelets are rapidly induced to express a cell associated form of the cytokine IL-1 (12). Agonists that stimulate its expression in vitro include adenosine diphosphate (ADP), collagen and thrombin, which are known to be present at sites of injury. Based on the importance of IL-1 in modulating endothelial function both in vitro and in vivo (5–8), we have now examined whether platelet derived IL-1 is present in a functionally relevant form that might thus provide an important early signal for the activation of the endothelium at sites of inflammation.

Materials and Methods

Platelet Preparation. Washed platelets were prepared from freshly drawn venous blood treated with 1/10 volume of citrate anticoagulant as previously described (12). Platelet preparations were contaminated with less than 0.1% red blood cells and no obvious (less than 0.01%) mononuclear cell contamination was observed. Washed platelet preparations were activated with 0.5-1 U/ml thrombin prepared from human plasma (Sigma Chemical Co., Poole, UK) for 2–4 min at room temperature.

[1] *Abbreviations used in this paper:* ADP, adenosine diphosphate; ELAM-1, endothelial leukocyte adhesion molecule 1; GM-CSF, granulocyte macrophage colony-stimulating factor; HUVEC, human umbilical vein; ICAM-1, intercellular adhesion molecule 1; SAVEC, adult saphenous vein.

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Endothelial Cell Culture. Human endothelial cells were derived from umbilical vein or adult saphenous vein by collagenase digestion as previously described (13), and grown on RPMI-1640 medium supplemented with 15–30 μg/ml endothelial growth factor (Sigma Chemical Co., 50 U/ml sodium heparin and 10% newborn (NBS) plus 10% FCS. Cells were routinely used between the second and fifth passages.

1 or 2 d before an experiment endothelial cells were plated on gelatin coated 24-well microtiter plates at a density of approximately $5 \times 10^4 - 10^5$ cells per well. Immediately before stimulation endothelial monolayers were washed 2–3 times in prewarmed serum free medium. Fig. 1 *A* demonstrates the purity of endothelial preparations by staining with two markers for endothelial surface antigens, antibody specific for gplla (CLB-HEC75; a kind gift from Dr. J.A. van Mourik, Central Laboratory of the Netherlands Red Cross Blood Transfusion, Amsterdam, The Netherlands) and Ulex Europaeus-1 lectin coupled to fluorescein isothiocyanate (ULEX-FITC; Sigma Chemical Co.).

Endothelial Cell Stimulation. Endothelial cells were cultured with $2–5 \times 10^7$ resting or thrombin activated platelets, supernatant from an equivalent number of activated platelets, thrombin (at a final concentration of 0.05–0.1 U/ml, and comparable to that added in the activated platelet preparations), the indicated concentrations of rIL-1β (specific activity 1.6×10^8 U/mg, kindly provided by Dr. P. Lomedico, Hoffman La Roche, Nutley, N.J.), or medium alone for 6–24 h at 37°C in a 5% CO_2 humidified atmosphere in a final volume of 1 ml RPMI-1640 with 10–15% FCS.

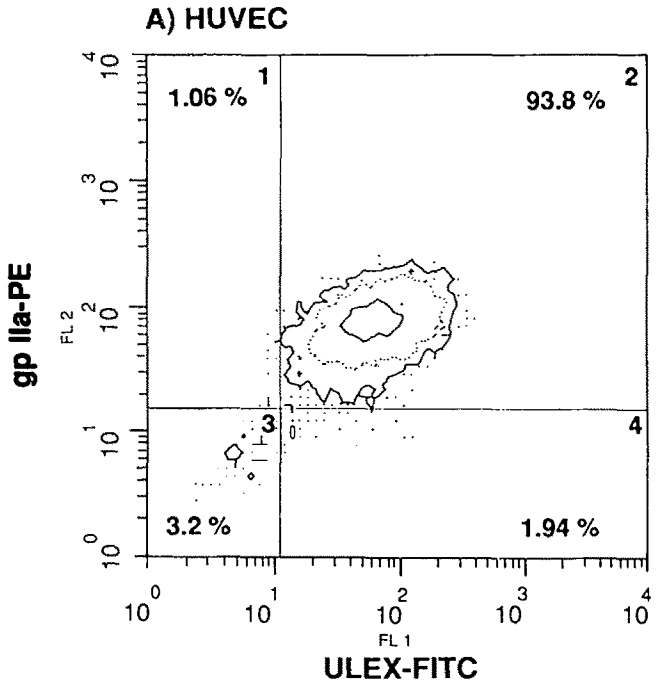
Anti-Cytokine Antibodies. In the experiments described in Fig. 1 *C* and 3 *B* and *D*, mouse mAb to human IL-1α (Dai Nippon, Japan; IgG1) was used at 4 μg/ml and goat antiserum specific for human IL-1β at a dilution of 1:800 (Immunex Corporation, Seattle, WA). Control irrelevant antibodies used were a 1:800 dilution of normal goat serum (Sigma Chemical Co.) and 5 μg/ml monoclonal mouse anti-human TNF (TNF-E, Genentech; IgG1). In Fig. 2 *B* a 1:100 dilution of polyclonal rabbit antiserum specific for IL-1α, IL-1β, or a combination of both (kindly donated by Immunex Corporation) were used to pretreat activated platelets before the addition to endothelial cell stimulation cultures and giving a final individual serum concentration of 1/1,000 in culture. Alternatively, polyclonal rabbit antiserum specific for IL-4 (used at 20 μg/ml) final concentration, Immunex Corporation) were used as control. Additional control antibodies used included goat antiserum specific for human IL-6 (kindly provided by Dr. L. Aarden, Central Laboratory of the Netherlands Red Cross Blood Transfusion, Amsterdam, The Netherlands) and rabbit antiserum to human transforming growth factor beta (TGFβ) generated in this laboratory by Dr. E. Abney.

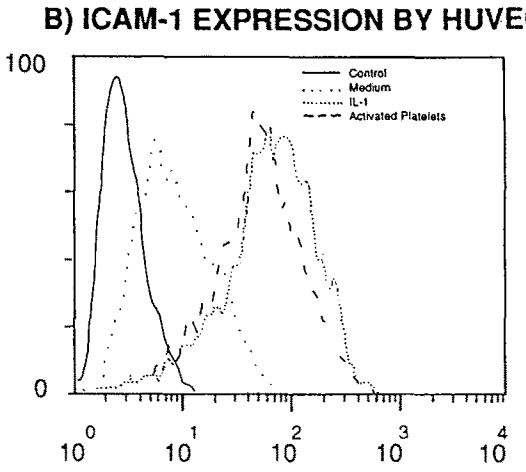
Immunostaining. Following culture endothelial cells were extensively washed in prewarmed HBSS and incubated with versene for 15–30 min at 37°C at which time they detached readily from the tissue culture plate, and were washed twice. Cells (approximately 10^5 per group) were stained for ICAM-1 expression with a 1:1000 dilution of mouse ascites of antibody 1H4, (kindly donated by Dr. A. Boyd, Melbourne, Australia), followed by 10 μg/ml goat antibody specific for mouse immunoglobulin (Ig) coupled to FITC (Southern Biotechnology Associates, Birmingham, AL). Alternatively cells were stained for the expression of ELAM-1 antigen with a 1:3000 dilution of ammonium sulphate precipitated ascites containing mouse antibody H18/7 (generously provided by Drs. M. Munro and M. Gimbrone, Boston, MA) and then the goat anti-mouse Ig FITC detection reagent. All cell groups were also stained with second layer reagent alone (goat anti-mouse Ig FITC). Control staining was performed with 100 μl culture supernatant from HB55 cells (American Tissue Culture Collection, Rockville, MD) containing antibody specific for HLA-DR class II antigen or a control mouse IgG2a FITC reagent (Becton Dickinson and Co.) used at 10 μg/ml. All staining was performed on ice in the presence of 0.02% sodium azide. Fluorescence analysis was performed by FACSCAN (Becton-Dickinson and Co., Mountain View, CA) and data is presented in the form of FACSCAN profiles (Fig. 1 *B* and *C*; fluorescent intensity, x axis versus cell number, y axis) or as mean fluorescence intensity values.

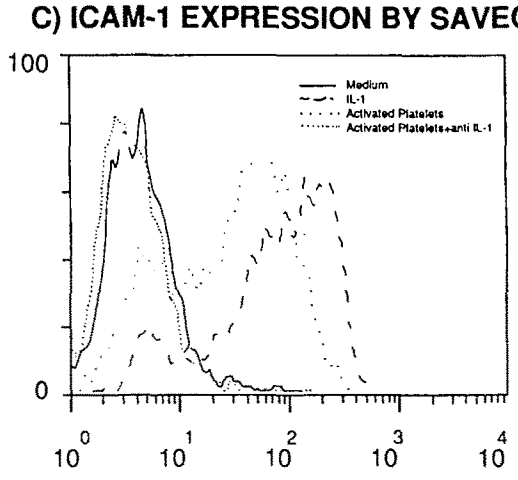
Cytokine Assays. Endothelial cell culture supernatants were harvested after overnight culture, filtered to eliminate platelet or endothelial cell contamination and either used immediately or frozen until used in the bioassays described below. IL-6 was measured using the B9 hybridoma cell line kindly provided by Dr. L. Aarden. Briefly, 2,000 B9 cells were cultured with either recombinant IL-6 (generously provided by Interpharm Laboratories, Israel; specific activity 5.65×10^6 U/mg) or 2- or 3-fold fold serial dilutions of the endothelial supernatants described above in triplicate wells to give a final volume of 200 μl in RPMI-1640 with 10% FCS. After 3 d incubation at 37°C the cells were pulsed with 0.5 μCi tritiated thymidine (^{3}H-thymidine) for the final 4 h of culture, harvested using a multi-well cell harvester (Skatron), and counted using a beta-plate system (Pharmacia, UK). The standard deviation of mean counts from triplicate wells rarely exceeded 10%. GM-CSF was assessed in an assay utilizing cells kindly provided by Genetics Institute, Boston, MA and with help from Dr. C. Haworth (Charing Cross Sunley Research Centre, London, UK). Briefly, 2×10^4 MO7-E cells were cultured for 3 d with endothelial derived supernatant or rGM-CSF (a kind gift of Dr. A. Krumwich, Behringwerke AG, Marburg; specific activity 4.5×10^7 CFU/mg) and measured for growth by incorporation of [^{3}H]thymidine during the last 6 h of culture. Data for both IL-6 and GM-CSF activity measurements are presented as units per ml of cytokine activity where the values of a minimum of 2, and up to 6, serial dilutions of endothelial supernatant were computed from a standard curve of recombinant cytokine and these values presented as the mean U/ml ± SD. Specificity of the bioactivity present in endothelial culture supernatants was ascertained by the addition of neutralising antibodies specific for IL-6 (goat anti-human IL-6, kindly provided by Dr. L. Aarden) and GM-CSF (rabbit antiserum generated by Drs. C. Haworth and E. Abney at Charing Cross Sunley Research Centre, London, UK), respectively.

Results and Discussion

Selective Induction of Adhesion Molecules on Endothelial Cells by Platelet-Derived IL-1. ICAM-1 is important for the adhesion of lymphocytes and monocytes to endothelium and its expression is upregulated both in vivo and in vitro on endothelial cells by recombinant (r)IL-1 (5, 14). In the current experiments, and as previously reported, both human umbilical vein (HUVEC) and adult saphenous vein (SAVEC) endothelial cell monolayers responded to rIL-1 by enhanced ICAM-1 expression (Fig. 1 *B* and *C*), as measured by FACSCAN analysis of specific binding of mAb 1H4. Thrombin activated platelets, when used to stimulate HUVEC or SAVEC, also induced a significant increase in surface ICAM-1 antigen expression. No significant difference was observed in the capacity of HUVEC or SAVEC to respond to platelets for enhanced ICAM-1 expression. Indeed while HUVEC are the most widely used model of endothelium, there may be







differences due to their foetal nature and for this purpose the two cell types were therefore compared throughout.

Our earlier data indicated that IL-1 was expressed predominantly by activated and not resting platelets (12). In the present study unstimulated platelets induced much lower increases in ICAM-1 expression on HUVEC (Fig. 2 *A*), although some variability was observed between experiments. In 7 experiments the fluorescent intensity ($\bar{x}$ ± SE of the mean) of staining for ICAM-1 on unactivated HUVEC was 33 ± 27, on cytokine induced HUVEC 198 ± 91, on HUVEC cultured with resting platelets 58 ± 27 or activated platelets 149 ± 42. Supernatants from activated platelets did not increase ICAM-1 expression on HUVEC, demonstrating a requirement for a membrane associated function expressed on activated platelets (Fig. 2 *A*). Finally, platelet agonists such as thrombin failed to stimulate ICAM-1 expression (Fig. 2 *A*) or influence ICAM-1 induction by rIL-1 (data not shown). A similar pattern of responsiveness was observed with SAVEC.

Earlier studies calculated that 1.8×10^7 platelets induced a response comparable to 1 U/ml rIL-1 in the D10.G4.1 functional assay for IL-1 (12). This agrees with the estimate made in the present study, where activated platelets were compared with rIL-1 for the capacity to enhance ICAM-1 antigen expression (Fig. 2 *A*). However this is a rough estimate since variation between platelet samples from different individuals was regularly observed (data not shown). This is in line with recent studies demonstrating similar individual variation in IL-1 production by peripheral blood derived monocytes from different donors (15).

To determine whether IL-1 or other platelet derived products mediated ICAM-1 induction, activated platelets were pretreated with antibodies to IL-1α and IL-1β before stimulation of HUVEC or SAVEC. The combination of both anti–IL-1 antibodies completely abrogated the capacity of activated platelets to increase HUVEC ICAM-1 levels above background (Fig. 2 *B*). A control antibody specific for IL-4 had no significant effect (Fig. 2 *B*), and in additional experiments normal rabbit antiserum or antibodies to IL-6 and TNF had no inhibitory effect on platelet induced ICAM-1 expression. Similar results were obtained with SAVEC (Fig. 1 *C*). The requirement for antisera to both IL-1α and IL-1β to obtain significant inhibition of platelet stimulated endothelial function was confirmed by the failure to obtain good inhibition with either alone (Fig. 2 *B*), and by titrating one antibody in the presence of a fixed concentration of the second (data not shown), and represents data reproduced with antisera from two separate sources (Fig. 1 *C* versus 2 *B*). This

Figure 1. Activated platelets enhance ICAM-1 antigen expression by human endothelial cells. Analysis of HUVEC (*A* and *B*) or SAVEC (*C*) stained: with control second layer reagent alone or for ICAM-1 expression on endothelial cells cultured overnight with medium, with rIL-1, with thrombin activated platelets or thrombin activated platelets pretreated with anti–IL-1 antibody as described in Materials and Methods. Fluorescence analysis was performed by FACSCAN (Becton-Dickinson and Co.) and data is presented in the form of FACSCAN profiles (*B* and *C*: fluorescent intensity, x axis versus cell number, y axis). (*A*) demonstrates the purity of endothelial preparations.

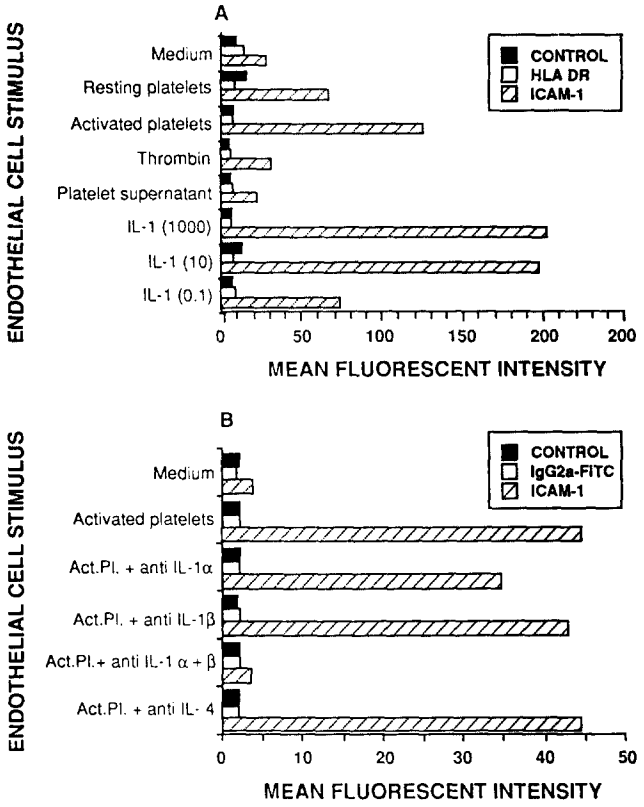


Figure 2. Activated platelets induce ICAM-1 expression on HUVEC in an IL-1 dependent manner. Endothelial cells were cultured with either 2×10^7 resting or activated platelets, platelet supernatants, thrombin, the indicated concentrations (in U/ml) of rIL-1β, or medium alone for 20 h at 37°C in a 5% CO_2 humidified atmosphere. In Fig. 2 *B*, 2×10^7 thrombin activated platelets were pretreated with medium alone or polyclonal rabbit antiserum specific for IL-1α, IL-1β, or a combination of both, or polyclonal rabbit antiserum specific for IL-4 before addition to confluent endothelial monolayers. HUVEC were then stained as described in Materials and Methods for ICAM-1 antigen expression (*hatched bars*). Control staining was performed for HLA-DR (Fig. 2 *A*; *clear bars*), or a control mouse IgG2a FITC reagent (Fig. 2 *B*; *clear bars*) and second layer reagent alone (control, Fig. 2 *A* and *B*; *solid bars*). Data are presented as mean fluorescent intensity staining evaluated by FACSCAN.

Table 1. *Activated Platelets Selectively Modulate Adhesion Molecule Expression by Cultured Endothelial Cells*

Endothelial cell		Mean fluorescent intensity expression of	
Source	Stimulus	ELAM-1	ICAM-1
		(n = 11)	*(n = 13)*
HUVEC	Medium	5.1 ± 1.6	27.8 ± 2.2
	IL-1	47.5 ± 18.9	195.3 ± 98.1
	Activated platelets	5.9 ± 3.0	102.3 ± 67.0
		(n = 3)	*(n = 3)*
SAVEC	Medium	5.7 ± 1.1	19.5 ± 7.8
	IL-1	57.8 ± 8.8	126.3 ± 23.9
	Activated platelets	27.5 ± 9.8	60.1 ± 9.7

HUVEC or SAVEC were cultured with medium, 1-100 ng/ml rIL-1β or 2×10^7 thrombin activated platelets for 6-8 h, at which time they were stained for the expression of ELAM-1 antigen and ICAM-1 antigen as described in Materials and Methods.

was surprising since earlier studies measuring platelet derived IL-1 activity in the D10.G4.1 T cell growth assay observed that mAbs specific for IL-1α and IL-1β inhibited 10–20% and 80–90% of platelet activity, respectively (12). The reason for this difference is unclear, but is most readily explained by differences in the antibody preparations themselves.

Plans to complete this study by showing the capacity of activated platelets to induce expression of a second IL-1 inducible adhesion molecule ELAM-1 (16), uncovered a surprising difference between HUVEC and SAVEC. Although SAVEC responded to activated platelets by the upregulation of ELAM-1 antigen in an IL-1 dependent manner, HUVEC selectively failed to upregulate the ELAM-1 antigen, as demonstrated in Table 1, despite clear enhancement of ICAM-1 measured in the same assays. The reason for this selectivity does not appear to represent presentation of insufficient IL-1 to HUVEC since increasing platelet number had no effect, nor was there any difference in the concentration of rIL-1 required to induce ELAM-1 or ICAM-1 expression on the same HUVEC population (data not shown). This suggests heterogeneity of function of endothelial cells derived from different sources and may represent differential sensitivity to other platelet products (inhibiting ELAM-1 induction in HUVEC but not SAVEC) which is currently under investigation.

Platelet-Derived IL-1 Stimulates Secretion of Cytokines by Cultured Endothelial Cells. The addition of purified rIL-1 in vitro stimulates the release of a number of chemotactic and cytokine activities by endothelial cells (5, 6). To test whether platelets modulated other parameters of endothelial IL-1 inducible function, secretion of IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) by endothelial cells was assessed following stimulation with activated platelets. Fig. 3 *A* and *C* demonstrate that activated platelets induced significant levels of bioactive IL-6 and GM-CSF production by HUVEC following 1 day of coculture as measured in the B9 and MO7E bioassays, respectively. The activity of supernatants derived from endothelial cells cultured with activated platelets and testing positive in IL-6 and GM-CSF bioassays were neutralized by addition of anti–IL-6 or anti–GM-CSF antibodies to the appropriate bioassay. Resting platelets induced significant but lower levels of these cytokines as demonstrated in both the GM-CSF and IL-6 bioassay data in Fig. 3 *A* and *C*. As predicted from the membrane bound nature of platelet IL-1, in all experiments performed supernatant derived from activated platelets had no cytokine inducing capacity. Supernatants derived from platelets cultured alone, in the absence

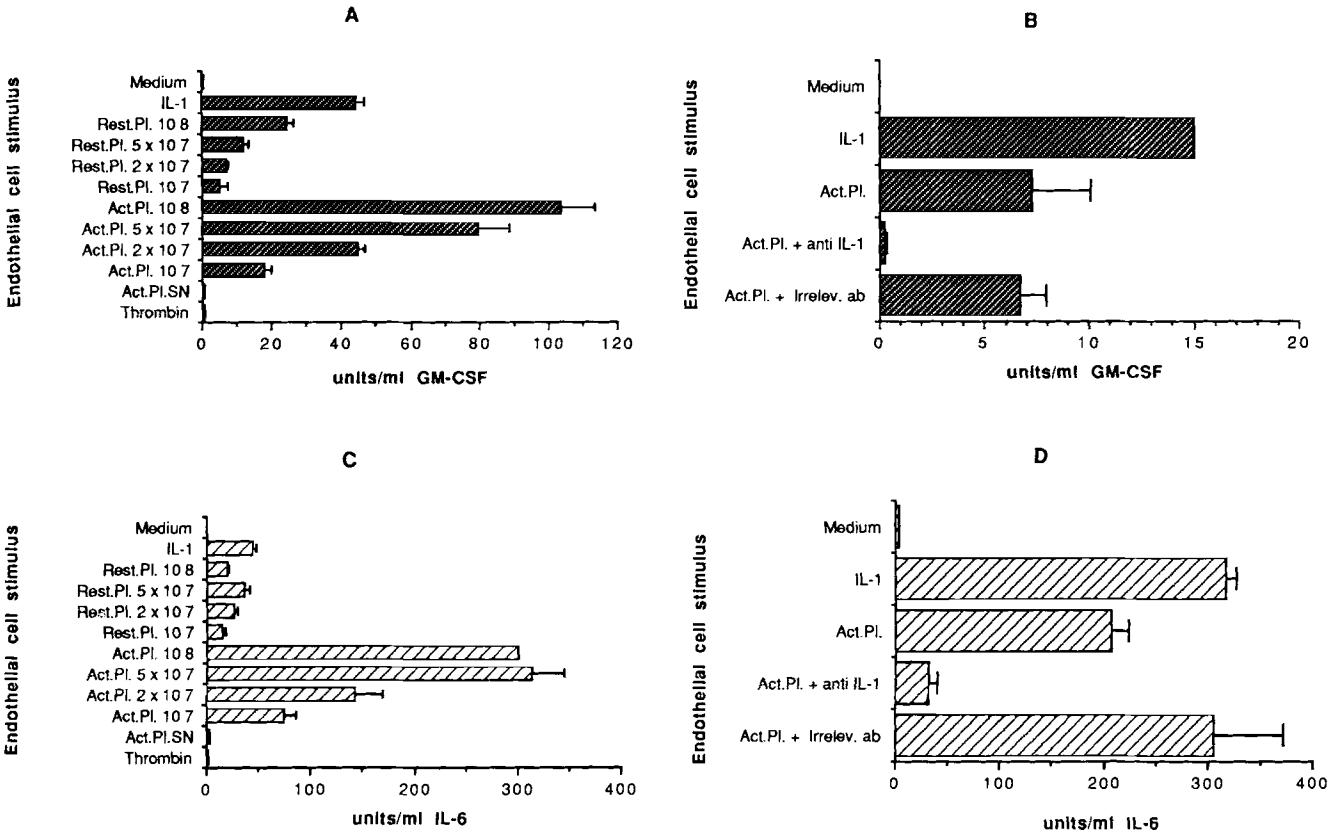


Figure 3. Platelet derived IL-1 stimulates secretion of the cytokines IL-6 and GM-CSF by cultured endothelial cells. (*A* and *C*) HUVEC were cultured with the indicated numbers of resting (Rest.Pl.) or activated platelets (Act.Pl.), platelet supernatant from an equivalent number of thrombin activated platelets (Act.Pl.SN), thrombin, 20 ng/ml rIL-1β, or medium alone for 24 hours to measure GM-CSF and IL-6 secretion as described in Materials and Methods. (*B* and *D*) SAVEC were cultured for 24 h with 10 ng/ml rIL-1β or 2 × 10^7 thrombin activated platelets which had been pretreated with irrelevant or anti-IL-1 antibodies and supernatants tested in the cytokine bioassays. Data is presented as mean U/ml of cytokine activity ± SD. Specificity of GM-CSF activity in endothelial cell (EC) culture supernatants was tested in the presence of specific antibody ($\bar{x}$ cpm ± SD): EC + medium 194 ± 40; EC + Act.Pl. 171,490 ± 3,675; EC+Act.Pl.+anti GM-CSF 8,190 ± 1,310; EC+Act.Pl.+control antibody/prebleed 154,135 ± 3310. Specificity of IL-6 bioactivity in endothelial culture supernatants was tested in the presence of specific antibody: EC+medium 12,260 ± 1,560; EC+Act.Pl. 55,215 ± 5,350; EC+Act.Pl.+anti IL-6 5,534 ± 1,390; EC+Act.Pl.+ control antibody/normal goat serum 59,880 ± 4,350.

of endothelial cells, failed to give a response in the cytokine assays (data not shown) demonstrating that secretion was due to endothelial cell activation. Activated platelets were also able to induce production of GM-CSF and IL-6 by adult human saphenous vein endothelial cells, and this response was abrogated by pretreatment of platelets with neutralizing antibodies to IL-1α and IL-1β, but not by irrelevant antibodies (Fig. 3 *B* and *D*).

The results presented in this paper demonstrate that activated platelets are potent stimulators of endothelial cells derived from both umbilical cord vein and adult saphenous vein for the increased expression of the adhesion molecule ICAM-1, and production of the cytokines IL-6 and GM-CSF. This is due to the expression of surface bound IL-1 activity since enhancement of ICAM-1 antigen and IL-6 and GM-CSF production could be abrogated by the pretreatment of activated platelets with antibodies specific for IL-1, but not irrelevant ones. Resting platelets were much weaker inducers of endothelial IL-1 inducible function. It is noteworthy that resting platelets frequently stimulated significant endothelial responses, particularly with respect to cytokine production. In this respect however, since no attempt was made to maintain unstimulated platelets in a resting condition it is probable that coculture with endothelial cells in itself induced some degree of platelet activation. Finally neither supernatant derived from thrombin activated platelets or thrombin itself induced responses from the cultured endothelial cells. This is in agreement with our previously published data that platelet IL-1 bioactivity is present in a cell associated, but not secreted from, and on activated rather than unstimulated platelets (12).

Failure to stimulate expression of the IL-1 inducible adhesion molecule ELAM-1 on HUVEC, but not SAVEC, demonstrated a surprising heterogeneity in the function of endothelial cells derived from different sources, pointing perhaps to a selective capacity of platelets to modulate endothelial cell function. A recent immunohistological study, supporting the concept of heterogeneous expression of ELAM-1 by different endothelia, demonstrates that vascular expression of ELAM-1 occurs at cutaneous sites rather than noncutaneous ones (17).

Following activation, platelets express or release a variety of mediators in addition to IL-1 including transforming growth factor beta, thrombospondin, β2 thromboglobulin, and

platelet factor 4 (18–21). These may variously suppress or enhance the response of endothelial cells to cytokines and a possible role for selective downregulation of ELAM-1 but not ICAM-1 on HUVEC by other platelet products is currently under investigation. It is also likely that platelet products other than IL-1 are also involved in enhancing endothelial cell stimulation by platelet derived IL-1, since on occasion activated platelets stimulated as high or higher levels of cytokine production than optimal concentrations of exogenous rIL-1. Nevertheless, although other products may enhance the effects of platelet delivered IL-1 with respect to endothelial production of cytokines (or perhaps downregulate the expected expression of ELAM-1), it is clear that these platelet induced responses necessarily involve the expression of IL-1 since they are completely inhibited in the presence of anti-IL-1α and β antibodies (Fig. 2 *B* and 3 *B* and *D*).

These data support our original hypothesis that platelets may provide an important initiating signal for the inflammatory response via their expression and delivery of IL-1 to vascular endothelial cells following injury. Platelets are ideally suited for such a role since they are targeted to local sites of endothelial injury, act quickly by expressing IL-1 within minutes of activation and deliver a localised signal, as all platelet delivered IL-1 appears membrane associated. Thus in addition to their crucial role in haemostasis, platelets may also be important in regulating inflammatory functions of the vascular endothelium, and may conceivably affect other cells with which they interact (for example monocytes, neutrophils, fibroblasts and smooth muscle), via their expression of proinflammatory cytokines such as IL-1. The demonstration that platelet derived IL-1 has functional consequences on vascular endothelium adds to the growing body of data implicating platelets as critical inflammatory components (22, 23).

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