

Anti-platelet factor 4/heparin antibodies from patients with heparin-induced thrombocytopenia provoke direct activation of microvascular endothelial cells

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Abstract

Heparin-induced thrombocytopenia (HIT) is a serious complication that occurs in ~1–5% of patients treated with heparin and may be associated with severe thrombotic events. HIT is mediated by antibodies directed mostly to epitope(s) formed by complexes between heparin or other anionic mucopolysaccharides and platelet factor 4 (PF4). Anti-PF4/heparin IgG antibodies from six patients with HIT were affinity purified and assessed for interaction with human microvascular and macrovascular endothelial cells (EC). The antibodies directly activated primary cultures of human bone marrow microvascular EC (HBMEC) and SV40 immortalized HBMEC (TrHBMEC) only in the presence of PF4, but did not activate macrovascular human umbilical vein EC (HUVEC) under the same conditions. These antibodies were found to bind to TrHBMEC through the F(ab)₂ portion of the anti-PF4/heparin IgG. TrHBMEC activation was characterized by an augmented release of IL-6, von Willebrand factor, soluble thrombomodulin, and by an elevated expression of the adhesion molecules P-selectin, E-selectin and vascular cellular endothelial molecule-1 to different degrees. Enhanced monocyte adhesion to PF4/heparin antibody-treated TrHBMEC (33–72% adhesion) was also observed. None of these effects occurred with unstimulated HUVEC. However, pre-treatment of HUVEC with tumor necrosis factor- α resulted in the same changes observed with microvascular EC exposed to the HIT antibodies. Our findings indicate that anti-PF4/heparin antibodies directly activate microvascular EC while interaction with macrovascular EC requires pre-activation. These results may explain some of the specific clinical manifestations in HIT.

Introduction

Heparin therapy can result in two types of thrombocytopenia. One is a mild, transient, non-immune disorder and generally has no adverse clinical consequences. The other, known as heparin-induced thrombocytopenia (HIT), is the potentially

serious and Ig-mediated type that carries a risk of serious thromboembolic events (1–6). It is widely accepted that HIT is mediated by antibodies directed against complexes that form between heparin or other anionic mucopolysaccharides

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and platelet factor 4 (PF4). The antibodies that target PF4/heparin complexes have been detected in the plasma of HIT patients (7–9). These antibodies bind the heparin/PF4 complex via the Fab portion of the antibody and cause platelet activation via binding of the Fc portion of the antibodies to platelet FcγRII receptors (CD32) (10–12). In addition, sera from patients with HIT and thrombosis contain antibodies that react with endothelial cells (EC) (13). When incubated with human umbilical vein EC (HUVEC), these sera deposited higher than normal amounts of IgG and/or IgA on the HUVEC and stimulated the production of tissue factor (13). IgG antibodies purified from HIT sera were shown to specifically interact with HUVEC that express heparin-like glycosaminoglycans, only in the presence of PF4 (14). This binding reaction was inhibited by excess heparin but not by anti-FcγII. The FcγII protein is not expressed by HUVEC (14). These observations suggested that immune injury to EC might play a role in the development of thrombosis in patients with HIT (13). While a recent study failed to show a direct activation of HUVEC by HIT antibodies, in the presence of washed platelets the sera from patients with HIT induced the expression of E-selectin, intracellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1 and tissue factor, and the release of IL-1β, IL-6 and PAI-1 (15). The presence of platelets seemed to be an indispensable requirement for HIT antibodies to induce HUVEC activation, since the activation could be inhibited by SR121566A, a potent and selective inhibitor of the platelet fibrinogen (GPIIb-IIIa) receptor, and by apyrase, which blocks the effects of released ADP (15).

It was recently shown that plasma from patients with another platelet-mediated thrombotic disease, thrombotic thrombocytopenic purpura (TTP), induce cellular damage and apoptosis of cultured microvascular EC but not macrovascular EC (i.e. HUVEC) (16,17). Endothelial damage was observed mainly in microvascular EC isolated from organs affected by TTP (brain, skin, kidney), while EC from organs spared by the disease (lungs, liver) were not sensitive to the TTP plasma (16). Since the thrombotic phenomena in HIT occur both in large vessels (arteries and veins) as well as in capillaries (skin), we decided to investigate the interaction of HIT antibodies with cultured micro- and macrovascular EC. For our study, we used microvascular human bone marrow EC (HBMEC) and compared their reaction to the HIT antibodies with the reaction of HUVEC. Our findings demonstrate that HIT antibodies directly bind and activate microvascular EC, while interaction with macrovascular EC is observed only following exposure of the HUVEC to either tumor necrosis factor (TNF)-α or, as has been previously shown, to washed platelets (15).

Methods

Patients and controls

Six patients, three males and three females, median age 63 (range 56–70), who were treated with heparin for deep venous thrombosis (DVT) (two patients) or coronary artery bypass graft operation (two patients) or cardiac catheterization (two patients) were included. All the patients showed marked reduction of their platelet counts (by >50%) during heparin therapy with counts <100×10⁹/l for no other reason than HIT.

All of these patients were evaluated by the hematology service to rule out other causes of thrombosis such as TTP, antiphospholipid antibody, Factor V Leiden, etc. In addition, all patients were tested by both the functional serotonin release assay and immunologic ELISA test for antibodies to heparin to confirm that the patients had heparin antibody-induced thrombosis. Five patients also developed HIT-related thrombotic events, one developed a myocardial infarction, two patients developed DVT and two had a significant extension of their DVT. None of the patients had skin manifestations. All the patients were positive for HIT antibodies by an ELISA (HPIA; Stago, Asnieres, France) and five were positive by the platelet serotonin-release assay, performed as previously reported (15). Ten age-matched healthy individuals served as controls and their plasma showed no HIT antibody by either of the two assays.

EC

HBMEC were used as primary cultures or as a cell line after transfection with a construct containing the SV40 large T antigen (TrHBMEC). These cells were prepared as previously described (18). Another unrelated line of SV40 transformed human bone marrow microvascular EC, a kind gift from Dr S. Rafii (Weill Medical College of Cornell University, New York) was used for comparison (19). Primary cultures of HUVEC derived from five umbilical cords for each experiment were used as macrovascular EC (20). For all the EC activation studies, the cells were preincubated for 8 h in the presence of 1% FCS prior to testing.

Affinity purification of anti-PF4/heparin antibodies and F(ab)₂ preparation

Plasma from the HIT patients, containing anti-PF4/heparin IgG, was used for affinity purification of IgG antibodies reactive with PF4/heparin complexes (HIT antibodies) in a three-step procedure. A column of PF4/heparin was established by incubating PF4 with heparin–Sepharose beads (Pharmacia Biotech, Norden, Sollentuna, Sweden). These beads were incubated with highly purified PF4 (50 μg/ml) in PBS with rotation at 4°C overnight. The beads were then loaded in an empty column and extensively washed with PBS to remove any unbound PF4. Total IgG fractions purified from the HIT patients' plasma by passage over Protein G (Pharmacia) were then loaded on the PF4/heparin column. IgG enriched in anti-PF4/heparin antibodies was eluted by glycine–HCl (0.2 M, pH 2.5), neutralized with Tris buffer, dialyzed extensively against PBS and concentrated. The eluate was used as enriched affinity-purified anti-PF4/heparin antibodies. Control IgG that were used in the study are: HIgG, IgG purified from plasma of healthy individual by passage sera over a Protein G column; IHIgG, derived from HIT sera inactivated with HUVEC in the absence of PF4 which did not bind or activate HUVEC; TA, SR-2 IgG mAb originated from a patient with Takayasu's arteritis (TA; a disease only affecting large arteries) that bind and activate exclusively large vessel EC (HUVEC) and not TrHBMEC (EC from microvascular origin) (20).

F(ab)₂ was prepared by dialyzing IgG fractions against 100 mM Na-acetate buffer, pH 4.0, and digesting them with pepsin (2% w/w; Sigma, St Louis, MO) at 37°C for 18 h (20). Fc fragments and any remaining traces of undigested IgG

were removed by binding to a Protein A column (Pharmacia). The efficiency of the digestion was confirmed by 10% SDS-PAGE.

Anti-PF4/heparin binding to EC

Binding of anti-PF4/heparin antibodies to EC was determined by cyto-ELISA using unfixed EC cultures, maintained in 96-well tissue-culture plates (Nunc, Dalton, Denmark) for 48 h. Following blocking with 3% BSA, the cells were incubated for 4 h with PF4 (25 µg/ml), a concentration which was found to be optimal for maximum binding. Intact anti-PF4/heparin antibodies or their F(ab)₂ and Fc fragments or a control IgG were added to the cells for 4 h, and probed with goat anti-human-IgG conjugated to alkaline phosphatase (Jackson Research, West Grove, PA) and then exposed to the appropriate substrate (Sigma). In another set of experiments HIT antibodies were added simultaneously with PF4 to TrHBMEC and the binding was determined as described above. In all these experiments, a monoclonal anti-endothelial cell antibody derived from a patient with TA, HIgG and IHlgG were used for comparison. The preparation and characterization of this mAb has been previously reported (20).

Markers of endothelial activation used in the study

Various proteins secreted or expressed by activated EC were measured. These included IL-6, von Willebrand factor (vWF), thrombomodulin (TM), and the adhesion molecules P-selectin, E-selectin, ICAM-I and VCAM-I.

The concentration of IL-6 in the culture fluid of EC after 24 h exposure to the affinity-purified anti-PF4/heparin was measured by commercial kits, according to the manufacturer's specifications (R & D Systems, Minneapolis, MN).

The vWF concentrations in the above culture fluid was determined by ELISA. Plates coated overnight at 4°C with mouse anti-vWF (2 µg/ml) in PBS were blocked with 3% BSA. The tested culture fluids were added for 4 h at room temperature. vWF in the culture fluid was detected by rabbit anti-vWF antibody, and probed by goat anti-rabbit alkaline phosphatase and appropriate substrate. The anti-vWF antibodies were kindly provided by Dr Aida Inbal (Department of Hematology, Sheba Medical Center, Tel Hashomer, Israel).

TM in the cell culture fluids or the TM present in lysates of EC that had been preincubated with anti-PF4/heparin IgG was determined by a capture ELISA (Asserachrom ELISA kit; Diagnostica Stago, Asnieres, France). The ELISA was performed with anti-TM precoated plates, according to the manufacturer's instructions. A 1:3 dilution was used for cell culture fluids, the concentrations were calculated in relation to the standard curves and means of the duplicates were taken.

For the detection of adhesion molecules on EC treated with anti-PF4/heparin antibodies the cells were grown in 96-well plates and preincubated with PF4. Then the cells were incubated with different concentrations of purified anti-PF4/heparin antibody or with a fixed concentration of anti-PF4/heparin antibody for different time periods (4, 8, 16 and 24 h). The cultured cells were washed and incubated with PBS containing 0.2% Triton X-100 in order to permeabilize the cell membrane (21). The plates were blocked with 3% ovalbumin, and incubated for 1 h with 1 µg/ml of a mixture of biotinylated mouse anti-human antibodies directed against

E-selectin, P-selectin, ICAM-I and VCAM-I (PharMingen, San Diego, CA). The cells were then exposed to streptavidin-alkaline phosphatase and the appropriate substrate. The results were expressed as OD units after the subtraction of blank values.

Adhesion of monocytes to activated EC

U937 (a human monocyte macrophage-like cell line) cells were pretreated with heat-aggregated γ-globulin for 30 min at 37°C (to block Fc receptor binding) and labeled with 0.5 µCi/ml of [³H]thymidine (Amersham International, Little Chalfont, UK) for 24 h. Adhesion of the radiolabeled monocytes to EC was studied after exposure of EC to PF4 followed by the addition of different concentrations of the anti-PF4/heparin antibodies. Non-adherent U937 were removed by washing and the adherent cells were lysed with formic acid. Radioactivity associated with adherence of U937 to the EC was quantified by β-scintillation spectroscopy. The results were expressed as percent of added U937 cells that had adhered and are presented as mean ± SD from three replicate wells (22).

A separate set of experiments was designed to test the prevention of monocyte adhesion by antibodies directed to the adhesion molecules. Accordingly, a mixture of monoclonal anti-VCAM-I, E-selectin and P-selectin antibodies (PharMingen) (20 µg/ml) were added to the EC following preincubation with the anti-PF4/heparin antibodies (25 µg/ml for 16 h), and the assay was continued as described above.

Statistical analysis

Statistical analysis was performed by using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

The anti-PF4/heparin antibodies bind directly to microvascular EC

Figure 1 shows the binding of affinity-purified anti-PF4/heparin IgG from six patients with HIT to TrHBMEC. Significantly increased binding ($P < 0.001$) was observed in comparison to binding of control IgG: HIgG, from healthy donor, $P > 0.05$; IHlgG, derived from HIT sera inactivated with HUVEC in the absence of PF4, $P > 0.05$; or TA, a mAb from a patient with TA which activates exclusively HUVEC and not EC from microvascular origin (20), $P > 0.05$. Binding of the anti-PF4/heparin antibodies took place through the F(ab)₂ portion of the IgG (Fig. 1), since binding of the intact anti-PF4/heparin and the F(ab)₂ fragment were each significantly greater than binding of the Fc portion of the antibody to the cells ($P < 0.001$). The binding of the HIT antibodies to TrHBMEC was dependent on their concentration (Fig. 2). The maximal binding of anti-PF4/heparin to TrHBMEC differed from one patient to another (Fig. 2). The values ranged between 0.737 ± 0.035 OD units for patient #6 and 1.296 ± 0.097 OD units for patient #2 measured at 405 nm and at 25 µg/ml of anti-PF4/heparin IgG. A separate set of experiments confirmed the significant binding of anti-PF4/heparin antibodies to primary cultures of untransformed bone marrow microvascular EC

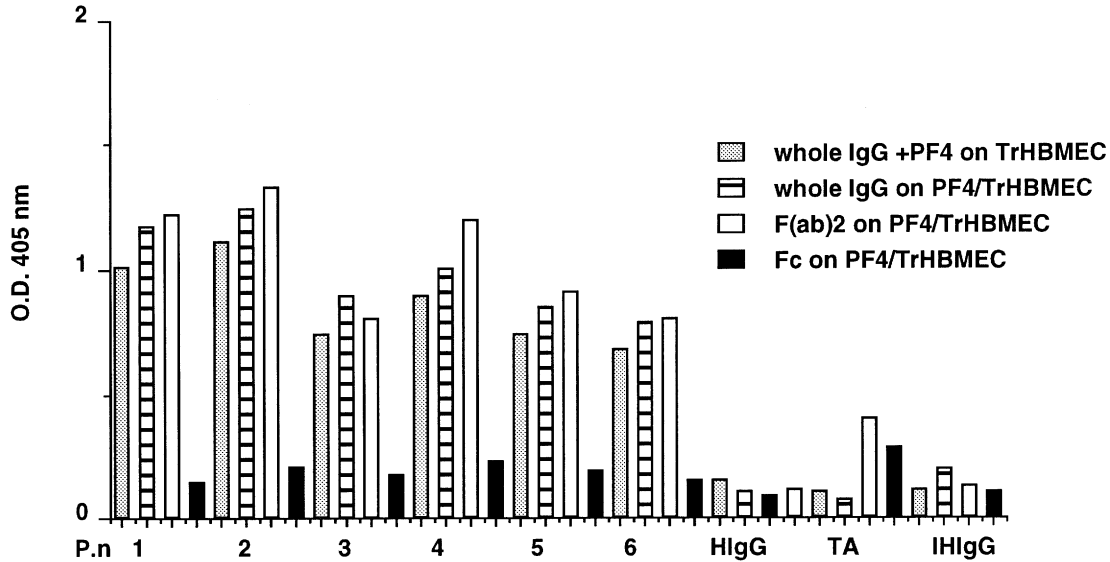


Fig. 1. Binding of affinity-purified anti-PF4/heparin antibodies to microvascular EC. Binding of anti-PF4/heparin whole molecule, the F(ab)₂ and Fc portions to TrHBMEC. Data are presented as absorption units in a capture ELISA at OD 405 nm and are pooled from two separate experiments. In both panels, samples 1–6 are antibodies from HIT patients. TA is the IgG mAb from a patient with TA that binds to macrovascular EC preferentially. HIgG is pooled control IgG from healthy donors which does not bind PF4/heparin.

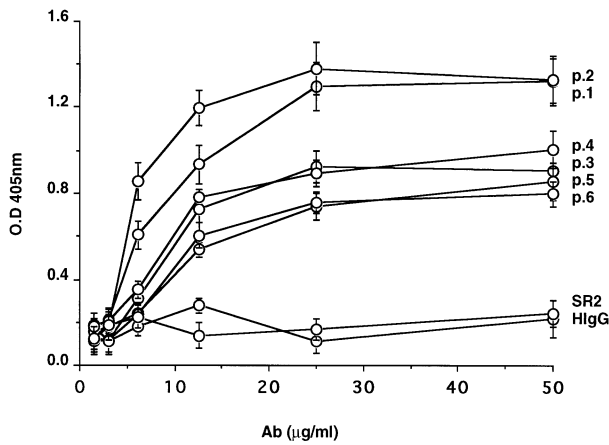


Fig. 2. Dose-dependent binding of anti-PF4/heparin antibodies to microvascular EC. Affinity-purified anti-PF4/heparin antibodies were tested for binding to TrHBMEC at different concentrations (range 0–50 µg/ml). Data are presented as mean ± SD of values in two separate experiments pooled together for absorption (expressed in OD units) in a capture ELISA at 405 nm. TA and HIgG are described in Fig. 1.

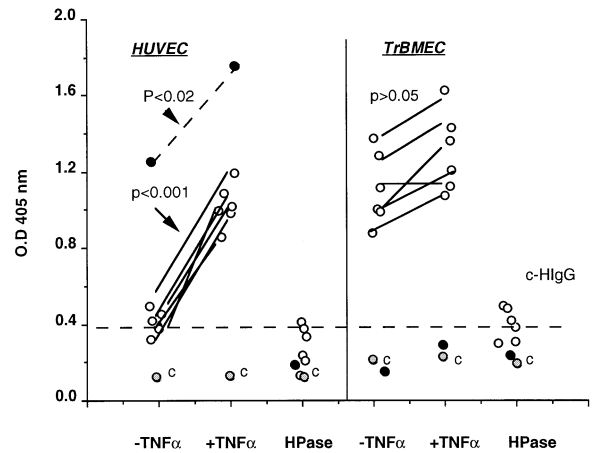


Fig. 3. The binding of anti-PF4/heparin to macrovascular and microvascular EC: effects of TNF-α and heparinase. Left panel, macrovascular EC (HUVEC). Right panel, microvascular EC (TrHBMEC). HUVEC did not bind significant amounts of anti-PF4/heparin IgG unless they were pretreated with TNF-α. TrHBMEC bind significant amounts of anti-PF4/heparin IgG when unactivated and TNF-α treatment does not increase binding. Heparinase treatment of both cell types, which eliminates EC membrane heparan sulfate, results in inhibition of the binding of anti-PF4/heparin IgG in all cases. Data are presented in OD units at 405 nm.

(HBMEC) or to another, independently derived line of SV40 transformed HBMEC (data not shown).

All anti-PF4/heparin antibodies failed to bind to unactivated HUVEC and significant binding occurred only following pre-stimulation of the HUVEC with TNF-α (Fig. 3). In comparison, the significant binding of the HIT antibodies to TrHBMEC was not altered by pretreatment of these cells with TNF-α (Fig. 3). Treatment of the HUVEC and TrHBMEC with heparinase, which degrades EC-associated glycosaminoglycans that are

the binding sites for PF4, abolished the binding of the anti-PF4/heparin antibodies to the cells (Fig. 3).

Affinity-purified anti-PF4/heparin antibodies bind differentially to TrHBMEC via PF4 molecules in a dose-dependent manner. The recognition of anti-PF4/heparin antibodies TrHBMEC was analyzed by direct binding, by preincubating the TrHBMEC with PF4 molecules before adding the Ig and

Table 1. Activation of microvascular EC upon exposure to anti-PF4/heparin

Patient no.	IL-6 (ng/ml)			vWF (OD 405 nm)	
	P4/TrHBMEC ^a	TrHBMEC ^b	HBMEC	PF4/TrHBMEC	HBMEC
1	>3000	2453	1872	1.423 ± 0.172	0.893 ± 0.087
2	>3000	2136	2192	1.234 ± 0.201	0.792 ± 0.099
3	>3000	1795	1289	1.378 ± 0.648	0.648 ± 0.078
4	>3000	2064	1724	0.891 ± 0.071	0.637 ± 0.057
5	>3000	2064	1724	0.891 ± 0.071	0.637 ± 0.057
6	2062	1306	1008	1.101 ± 0.057	0.438 ± 0.069
HlgG1	152	184	71	0.247 ± 0.077	0.092 ± 0.048
IHlgG1	201	168	118	0.201 ± 0.082	0.115 ± 0.053
SR2	387	303	201	0.157 ± 0.068	0.101 ± 0.039
PF4 alone	216	184	124	0.108 ± 0.072	0.095 ± 0.053

^aPF4/TrHBMEC preincubated with PF4 and then the tested antibodies were added.

^bTrHBMEC studied antibodies and PF4 were subjected simultaneously to TrHBMEC.

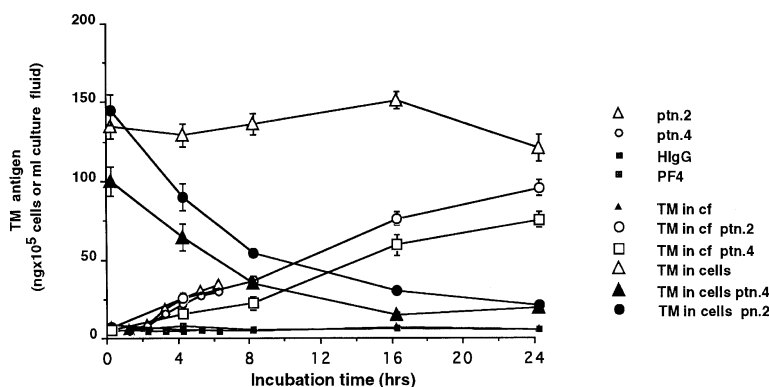


Fig. 4. TM expression by microvascular EC exposed to anti-PF4/heparin. TM levels in culture fluids from TrHBMEC cells exposed to 10 µg/ml anti-PF4/heparin antibodies from two HIT patients. Data are presented as mean ± SD of two separate experiments.

by adding the IgG in concert with PF4 molecules. The recognition of anti-PF4/heparin antibodies by HUVEC was analyzed by direct binding, by preincubating TNF- α -activated HUVEC with PF4 molecules followed by addition of anti-PF4/heparin IgG or by adding the studied IgG in concert with PF4 onto TNF- α -activated HUVEC.

It was found that anti-PF4/heparin antibodies bind TrHBMEC differentially and via PF4 molecules since no direct binding of anti-PF4/heparin IgG to TrHBMEC was detected unless the EC were preincubated with PF4 or the tested antibodies were added in concert with PF4 molecules. The binding of anti-PF4/heparin antibodies to PF4/TrHBMEC reached a plateau at a PF4 concentration of 20 µg/ml (OD at 405 nm ranged from 0.820 ± 0.079 up to 1.414 ± 0.119), $P < 0.001$ up to $P < 0.01$ for all the tested anti-PF4/heparin when compared to the negative binding to TrHBMEC by TA IgG, IHlgG or HlgG at the same experimental conditions. The binding of anti-PF4/heparin antibodies (10 µg/ml) when subjected in concert with PF4 (20 µg/ml) to TrHBMEC was similar (OD at 405 nm ranged from 0.689 ± 0.084 up to 1.205 ± 0.121), $P < 0.001$ up to $P < 0.02$ for all the tested anti-PF4/heparin when compared to the negative binding to TrHBMEC by TA IgG, IHlgG or HlgG under the experimental conditions employed. Similar results were obtained with untransformed HBMEC.

The binding of anti-PF4/heparin IgG to TNF- α -activated HUVEC was PF4 depending when these antibodies were added to PF4 preincubated HUVEC (OD at 405 nm ranged between 0.493 ± 0.069 up to 1.209 ± 0.112 at 25 µg/ml PF4), $P < 0.001$ up to $P < 0.02$ when compared to negative binding of TA IgG, IHlgG or HlgG at the same experimental conditions. Subjection of anti-PF4/heparin (10 µg/ml) simultaneously with PF4 at concentration of 25 µg/ml to TNF- α -activated HUVEC showed similar binding (OD at 405 nm ranged between 0.309 ± 0.083 up to 1.007 ± 0.118), $P < 0.001$ up to $P < 0.04$ when compared to negative binding of TA IgG, IHlgG or HlgG at the same experimental conditions.

Anti-PF4/heparin activates microvascular EC

PF4/TrHBMEC incubated for 16 h with affinity-purified anti-PF4/heparin antibodies (12 µg/ml) released significant amounts of IL-6 and vWF proteins (Table 1). Parallel results were obtained when the anti-PF4/heparin antibodies were added in concert with PF4 to TrHBMEC and induced release of IL-6 and vWF. This release was similar to the effect of stimulation of these cells with TNF- α (4 ng/ml) which resulted in the release of >3000 ng/ml IL-6. Smaller but significant amounts of IL-6 and vWF were released by the non-transformed HBMEC that were incubated with anti-PF4/heparin antibodies

under similar conditions (Table 1). Exposure of the TrHBMEC to control IgG or the mAb from the TA patient resulted in a much smaller and not statistically significant release of the two proteins (Table 1). The concentrations of IL-6 or vWF released by the EC upon exposure to PF4 (12–50 µg/ml) alone were negligible (data not shown).

The release of TM, another marker of endothelial activation, was evaluated with antibodies derived from two HIT patients (#2 and #4). Elevated levels of soluble TM were detected in the culture medium of TrHBMEC preincubated with PF4 (10 µg/ml) followed by anti-PF4/heparin antibodies (12 µg/ml) up to 24 h. The TM levels in the culture fluids increased gradually over time with significant TM levels already detectable after 3 h of incubation. A parallel decrease of cell-associated TM was observed with exposure of TrHBMEC to the two antibodies (Fig. 4). Control experiments using human IgG or PF4 alone revealed no significant changes in cell-associated or released TM (data not shown).

Incubation of TrHBMEC with anti-PF4/heparin affinity-purified antibodies also resulted in a significant expression of E-selectin, P-selectin and VCAM-I but not of ICAM-I (Fig. 5a). Similar results were observed with the non-transformed HBMEC (data not shown) although the expression of these molecules was somewhat lower (by 20–25%) in these cells. No significant ($P > 0.05$) expression of the adhesion molecules was observed when the TrHBMEC were incubated with PF4 alone or with PF4 added simultaneously with HIgG (Fig. 5a).

Kinetic studies showed a time-dependent enhancement of the expression of these adhesion molecules upon exposure to the HIT antibodies (Fig 5b).

Activation of microvascular EC by the HIT antibodies results in enhanced U937 monocyte adhesion

The enhanced expression of adhesion molecules upon exposure of the microvascular EC to anti-PF4/heparin antibodies was associated with increased adhesion of U937 cells. As shown in Fig. 6(a). All the HIT antibodies induced a significant increase in U937 adhesion to TrHBMEC, which was concentration dependent. The percent adhesion to TrHBMEC following exposure to PF4 and anti-PF4/heparin for 16 h was 32 ± 2 , 72 ± 2 , 41 ± 2 , 59 ± 3 , 27 ± 2 and $33 \pm 3\%$ for patients #1–#6 respectively ($P < 0.001$ – $P < 0.02$ when compared to adhesion following exposure of the EC to control IgG and the mAb from the patient with Takayasu). The augmented adhesion of U937 monocytic cells could be abrogated by exposing TrHBMEC first to a cocktail of anti-adhesion molecule antibodies directed against P-selectin, E-selectin and VCAM-I before the EC were incubated for 16 h with the anti-PF4/heparin antibody (Fig. 6b). Similar results were obtained with the second line of TrHBMEC (data not shown). Monocyte adhesion was not completely eliminated following pretreatment of TrHBMEC by the mixture of antibodies directed to adhesion molecules and this could be related to the possible presence of other adhesion molecules which were not blocked by the antibody mixture used (Fig. 6b).

Discussion

Anti-PF4/heparin antibodies are the hallmark of HIT and have been shown to induce platelet activation and thrombo-

cytopenia following their interaction with platelet Fc receptors (7–9). Under certain experimental conditions, the HIT antibodies were also found to be capable of activating cultured human EC (HUVEC), suggesting a mechanism for the occurrence of thrombosis in certain patients with HIT (10–14). However, it has been recently shown by one of us (J. M. W.) that HUVEC activation induced by HIT antibodies occurred only in the presence of washed human platelets, and was manifested by an increased release of cytokines and expression of adhesion molecules (15). It has been suggested that the HIT antibodies play a surrogate function in the immune injury of the vascular endothelium (15). Since the manifestations of HIT may be associated with thrombotic occlusions of both large blood vessels (veins and arteries) and microvessels, we studied the effects of purified anti-PF4/heparin antibodies derived from patients with HIT on microvascular EC. We used both primary cultures of HBMEC as well as two different lines of SV40-transformed HBMEC (TrHBMEC) which grow faster, remain well differentiated with stable phenotypic features during prolonged culture, are easier to handle and can be used over many passages.

The main finding of our study was the demonstration that the affinity-purified anti-PF4/heparin IgG antibodies were capable of binding to and causing a specific activation of microvascular EC, without any requirement for added platelets. In contrast, binding and activation of macrovascular HUVEC by the same antibodies were observed only following preactivation of HUVEC with TNF- α .

The binding of anti-PF4/heparin antibodies to microvascular EC and to preactivated macrovascular EC occurred only in the presence of PF4, a finding in agreement with previous reports (13,14). We showed that the anti-PF4/heparin antibodies bind EC via the F(ab)₂ portion of the Ig and not by the Fc portion. Moreover, binding of the HIT antibodies was also dependent on the presence of intact endothelial cell glycosaminoglycans, as degradation of these molecules with heparinase eliminated the binding. These molecules are involved in the binding of PF4 to EC (7).

The anti-PF4/heparin antibody caused a direct activation of microvascular EC (transformed and non-transformed) which was manifested by an augmented release of IL-6, vWF and soluble TM into the culture fluid. The release of TM directly correlated with decreased levels of TM bound to the EC membrane. These effects were not observed with macrovascular EC (HUVEC) treated with the same HIT antibodies. Interestingly, TM is a transmembrane proteoglycan receptor of thrombin which displays anti-thrombotic properties at the surface of the vascular endothelium. Increased levels of soluble TM are considered to reflect EC damage and have been observed in a variety of clinical disorders including TTP, diabetic microangiopathy, disseminated intravascular coagulation, systemic lupus erythematosus, adult respiratory distress syndrome and malignancy (23–32). A common finding in all these disorders is capillary thrombosis.

Microvascular EC exposed to anti-PF4/heparin antibodies showed increased expression of the adhesion molecules P-selectin, E-selectin and VCAM-I, while ICAM-I was not increased. The presence of increased adhesion molecules on the EC surface was associated with increased U937

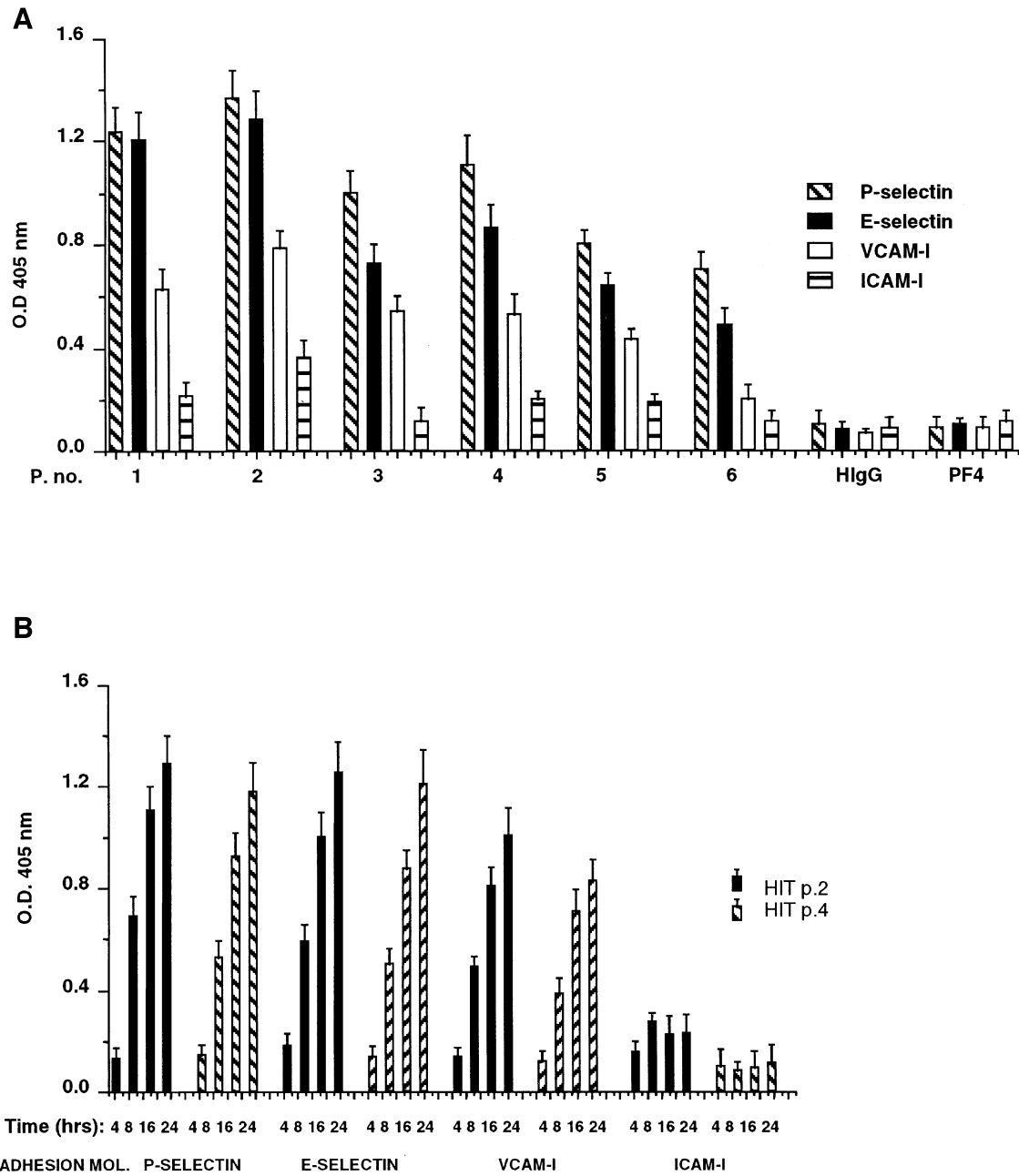


Fig. 5. Adhesion molecule expression by microvascular EC exposed to anti-PF4/heparin. (a) E-selectin, P-selectin, VCAM-I and ICAM-I expression on TrHBMEC following exposure to anti-PF4/heparin IgG (12 µg/ml) for 16 h. (b) Expression of adhesion molecules by TrHBMEC exposed to anti-PF4/heparin (12 µg/ml) for different time periods. Data are presented in OD units at 405 nm as mean ± SD of two separate experiments.

monocyte adhesion to the anti-PF4/heparin antibody-treated microvascular EC. The adhesion of monocytes could be nearly completely inhibited upon preincubating the EC with specific antibodies directed against these adhesion molecules. The phenomenon of enhanced expression of endothelial adhesion molecules had been previously observed with HUVEC exposed to HIT antibodies, but it depended on the presence of added platelets (15).

To demonstrate the specific avidity of the HIT antibodies for microvascular EC, we used a mAb previously isolated from a patient with TA (20). This IgG antibody was capable of directly activating cultured large vessel HUVEC, while it had no effect on TrHBMEC activation (20). TA is associated with accelerated atherosclerosis and thrombotic occlusions of large arteries. In contrast, it has been previously demonstrated that the plasma of TTP patients induced typical apoptotic

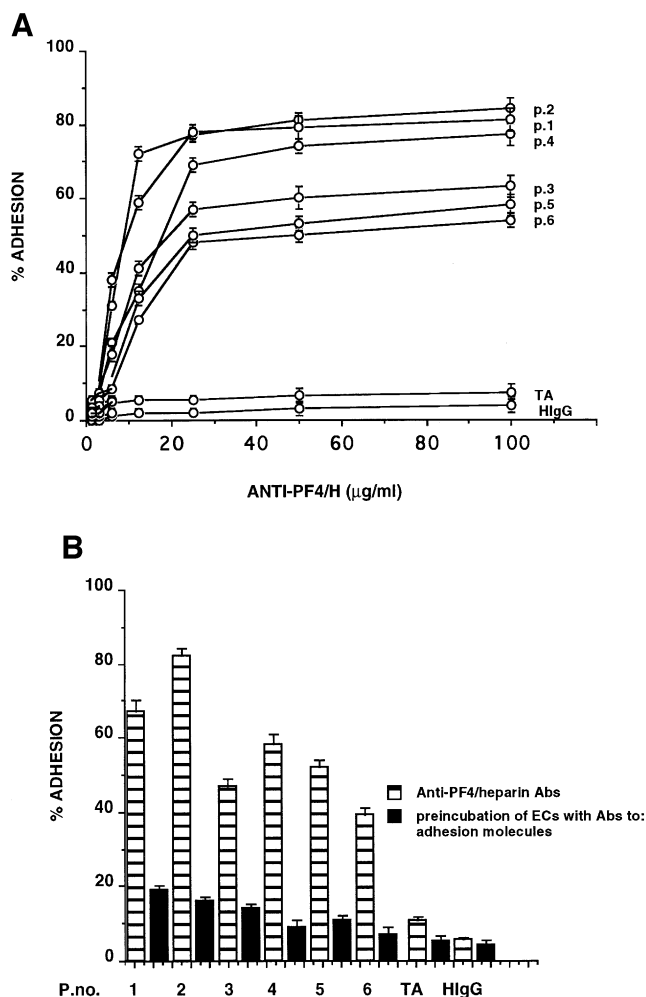


Fig. 6. Adhesion of monocytes to microvascular EC exposed to anti-PF4/heparin antibodies. (a) TrHBMEC were incubated with anti-PF4/heparin IgG at different concentrations for 16 h. Adhesion of [3 H]thymidine-labeled U937 monocytic cells was measured by scintillation spectroscopy. Data are expressed as percent of monocyte adhesion mean \pm SD of two separate experiments. (b) Percent of monocyte adhesion to TrHBMEC, preincubated with a combination of antibodies directed against E-selectin, P-selectin and VCAM-1, and then exposed to anti-PF4/heparin IgG. Data are expressed as percent of monocyte adhesion mean \pm SD of two separate experiments.

changes in certain microvascular EC, while no such damage occurred upon exposure of HUVEC to the same plasma samples (16). These two observations, taken together with our findings, provide further support to the concept that EC derived from different vascular beds have different characteristic features and can respond differently to injurious agents such as yet undefined components in the plasma of TTP patients. The concept of vascular bed-specific hemostasis and hypercoagulable states recently discussed by Rosenberg and Aird (33) is nicely illustrated by our findings. According to this concept, the endothelium integrates different extracellular signals and responds differently to the same endogenous or exogenous injurious agents in different regions of the vascular tree.

In summary, our findings shed additional light on the pathogenesis of thrombosis that occurs in association with heparin-induced thrombocytopenia. We demonstrated that activation of microvascular EC by the HIT antibodies is direct, while activation of large vessel EC occurs indirectly and is associated with platelet activation or the release of certain cytokines.

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Abbreviations

DVT	deep vein thrombosis
EC	endothelial cell
HBMEC	human bone marrow endothelial cell
HIT	heparin-induced thrombocytopenia
HUVEC	human umbilical vein endothelial cell
ICAM	intracellular cellular adhesion molecule
PF4	platelet factor 4
TA	Takayasu's arteritis
TM	thrombomodulin
TTP	thrombotic thrombocytopenic purpura
VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor

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