The platelet endothelial cell adhesion molecule-1 (PECAM1) contributes to endothelial barrier function

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Abstract In this study we have analyzed the role of the platelet-endothelial cell adhesion molecule-1 (PECAM1) in vascular barrier function. PECAM1 is an immunoglobulin gene superfamily member expressed by endothelial cells at the cell boundaries. Macromolecule permeability assays performed on cell monolayers that express native or transfected PECAM1, indicated that the molecule participates in the establishment and maintenance of vascular barrier function in vitro. This hypothesis was confirmed by the finding that in vivo injection of the specific monoclonal antibody directed against the murine vascular PECAM1 led to a detectable leakage of hepatic and renal blood vessels.

Key words: Vascular permeability; PECAM1; Endothelium; Adhesion molecule

1. Introduction

Vascular endothelium forms an active boundary between the bloodstream and the underlying tissues, retaining blood components within the intravascular space [1,2]. The integrity of the endothelial monolayer is a pre-requisite to dynamically control vascular permeability and is dependent on the presence of specialized junctions located between adjacent endothelial cells (EC) as well as on the anchoring of the basal side to subendothelial extracellular matrix [1,2].

A number of integral membrane molecules are implicated in the organization and maintenance of EC intercellular junctions: among them, cadherins [3,4] and integrins, mainly $\alpha 5\beta 1$ and $\alpha 2\beta 1$ [5]. The platelet-endothelial cell adhesion molecule-1 (PECAM1), also known as CD31, has been recently described as an additional molecule localized at the intercellular junctions and responsible for the formation of cell monolayers in vascular endothelium [6–9].

We have been interested in the role of PECAM1 as a cell adhesion molecule involved in lymphocyte activation and transendothelial migration [10,11]. In this study we demonstrate that both endogenously and ectopically expressed vascular PECAM1 is involved in maintaining EC monolayer integrity in vitro. We also provide evidence for a role of PECAM1 in the maintenance of vascular barrier functions in vivo.

2. Methods

2.1. Monoclonal antibodies (mAbs)

In the present study the following mAbs were used: the anti-PECAM1 mAbs M89D3 (formerly LAK1: 10, 12; see also Fig. 3); the anti-LFA3 (AICD58) obtained from Immunotech (Luminy, Marseille, France); the anti-murine PECAM1 (clone MEC13.3, IgG2a), the anti-murine ICAM1 (clone 3E2, IgG) and the anti-L3T4 (clone RM4-5, IgG2a) mAbs from Pharmingen (San Diego, CA). All the purified antibodies were extensively dialyzed and used at concentrations from 10 to 0.1 μg/ml in functional in vitro assays and a dose of 50 μg was used in in vivo studies.

2.2. Endothelial cell cultures and treatments

Human endothelial cells were isolated from umbilical vein (HUVEC: from now on EC) and cultured as described [13]. Cells were used within four passages. Exposure of EC (or PECAM1 transfectants; see below) to either intact or pepsin digested monoclonal antibodies ($F(ab')_2$, prepared according to Parham, ref [14]) was performed for 30 min at room temperature (to avoid any possible damage of the endothelial monolayer due to low temperature); cells were then washed three times and used. The ECV304 cell line [15] obtained from J.S. Pober (Yale University, Yale), was maintained in culture with DMEM containing 10% FCS.

2.3. PECAM1 transfectants

Lines D12P and P-ECV were made from NIH/3T3 murine fibroblasts or ECV304 human EC, respectively, with PECAM1 and neomycin resistance on the same plasmid. PECAM1 was subcloned into pcDNAI/Neo (Invitrogen, San Diego, CA) at the *XhoI* (5′) *NsiI* (3′) sites from the original pGEM7 vector (PECAM-1/pGEM7 (ref. [7]) was kindly provided by Dr. Peter Newmann, Blood Center of South-eastern Wisconsin, Milwaukee). Transfection was performed by calcium phosphate-DNA co-precipitation. Stable transfectants were selected by addition, after 48 h, of the neomycin analogue G418 (0.8 mg/ml). Neomycin resistant colonies were picked 10 days later, expanded and tested for PECAM expression by immunofluorescence using mAbs 5.6E (Immunotech) and M89D3. Mock transfected ECV304 and NIH/3T3 cells were used as negative controls.

2.4. Permeability assay

The Transwell cell culture chambers (polycarbonate filters, 0.4 μm pore size, Costar) were used as described [16]. Briefly, 5×10^4 EC, ECV304, D12P, P-ECV or mock transfectants were seeded onto the filters and grown to confluence. Some samples were exposed to various dilutions of the AICD58 or the M89D3 mAb (from 10 to 1 μg/ml). [^{125}I]Albumin (1 mCi/ml, NEN, Boston, MA) was added to the upper chamber; cold albumin (1.5 mg/ml) was added to the culture medium to minimize transcytosis (this concentration being saturating according to Simionescu and Simionescu (ref. [1]). Samples were taken from the

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Abbreviations: PECAM1: platelet endothelial cell adhesion molecule 1; EC: endothelial cells; PMSF: phenylmethylsulphonyl fluoride; SDS: sodium-dodecyl sulfate; DOC: deoxycolic acid; BSA: bovine serum albumine; PBS: phosphate buffered solution.

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lower chamber 1 h after addition of [^{125}I]albumin and the radioactivity was measured in a γ-counter (Packard, Sterling, VA). Results are the mean ± S.D. from 10 independent experiments with different EC lines and are expressed as albumin flux (pmol·cm^{-2}·s^{-1}) according to Fick's law ($Js = \Delta Cs/S\Delta t$).

2.5. Surface iodination and immunoprecipitation

Surface PECAM1$^-$ ECV304 endothelial cell line, PECAM1$^+$ D12P clone, PECAM1$^+$ transfected P-ECV cell line or mock transfected NIH/3T3 murine fibroblasts (E5) were iodinated (^{125}Iodine, 0.5 mCi/10^7 cells; NEN DuPont) using a lactoperoxidase catalyzed reaction [10]. Cells were then lysed in 1% NP-40 lysis buffer containing 10 μM pepstatin, 10 μM aprotin, 10 μM leupeptin, 10 μM antipain, 10 μM phenylmethylsulphonyl fluoride (Sigma). For immunoprecipitation, mAbs were coupled to protein-G sepharose; then 100 μl (100 μg protein) of precleared cell lysate were incubated with the pellet beads for 12 h at 4°C under rotation. Beads were washed twice and samples eluted at 50°C for 20 min. After separation by SDS electrophoresis (12.5% gel) the gel was dried and autoradiography performed at −70°C with appropriate films (NEN DuPont).

2.6. In vivo experiments

Balb/C mice (n = 6; Charles Rivers Italia, Como, Italy) were anesthetized with ether. The liver was perfused with 4% albumin in Evans blue through the inferior vena cava and the perfusate drawn from the portal vein according to Branster and Morton [17]. The right kidney was perfused through the renal artery as previously described [18]. Fifty micrograms of the anti-murine PECAM1, or the anti-murine ICAM1 or the anti-L3T4 mAbs were injected into the vena cava or the renal artery 15 min before the beginning of dye perfusion. Perfusion with saline alone or Evans blue alone was also performed. Both liver and kidney were then washed with saline, immediately removed and homogenized with buffered phosphate solution, pH 7.4. Samples were centrifuged and the supernatants recovered and treated with 10% deoxycolic acid (DOC, sodium salt monohydrate, Sigma) in saline. Dye presence was evaluated by spectrofotometric analysis (Pye Unicam, SP6-550, Cambridge, UK) at 540 nm wavelength.

3. Results

3.1. Contribution of PECAM1 to the maintenance of vascular barrier function

Confluent monolayers of EC were analyzed for their ability to prevent the trans-endothelial flux of a radioactive macromolecule, [^{125}I]albumin, in the presence or absence of the blocking anti-PECAM1 mAb M89D3. Fig. 1A shows that [^{125}I]albumin flux through EC monolayers was enhanced by preincubation with the specific anti-PECAM1 mAb; albumin flux increased from 0.2 ± 0.05 pmol·cm^{-2}·s^{-1} when EC were exposed to culture medium alone, to 1.2 ± 0.08 pmol·cm^{-2}·s^{-1} when they were treated with the M89D3 mAb. The effect of the anti-PECAM mAb was also detectable using the F(ab')$_2$ fragment and showed a clear dose-dependence (Fig. 1A). Similar effects were observed using the Fab' of the M89D3 mAb (not shown). A mAb directed against LFA3, a molecule whose EC surface density is comparable to PECAM1 (not shown), did not affect albumin flux (Fig. 1A). A control anti-human ICAM1, whose surface expression was lower than that of LFA3, did not exert any detectable effect (not shown); conversely, co-incubation of EC with both M89D3 mAb and an anti-E-cadherin or an anti-N cadherin mAb increased the permeability up to 1.7 ± 0.05 pmol·cm^{-2}·s^{-1} (not shown). Baseline transit of albumin in this assay (0.2 ± 0.05 pmol·cm^{-2}·s^{-1}) was consistently low and

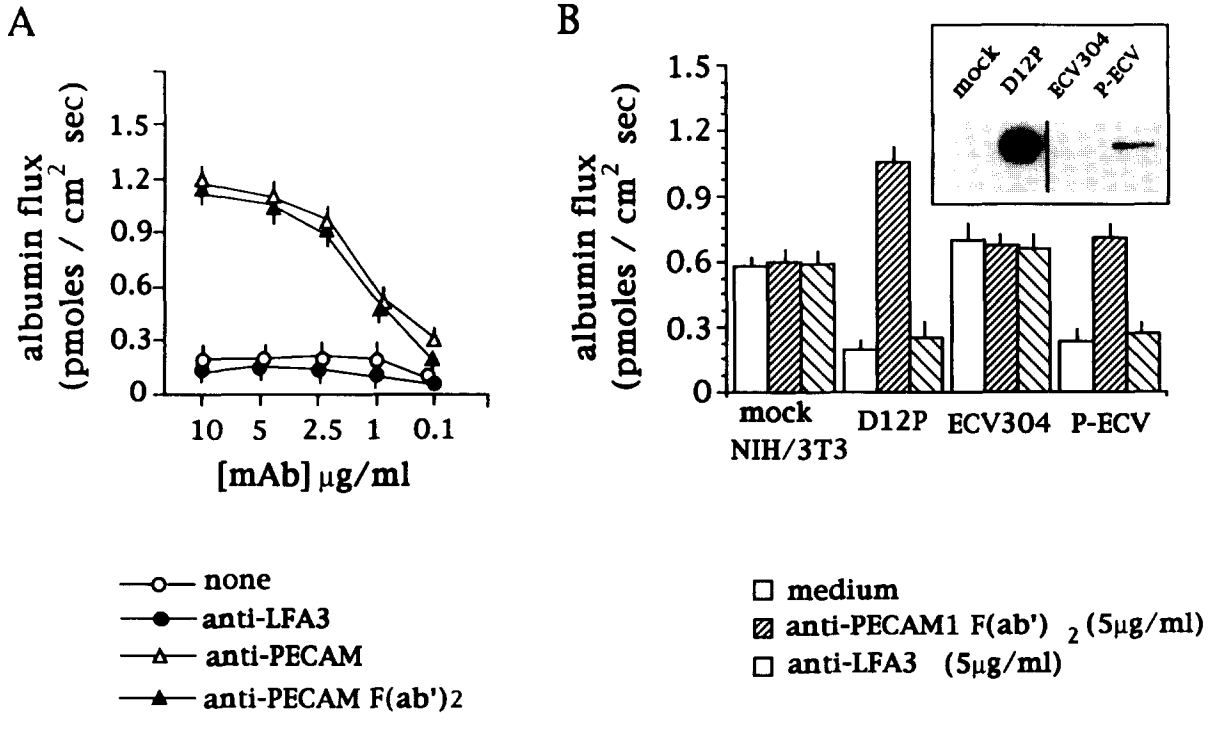

Fig. 1. [^{125}I]Albumin transit through endothelial and non-endothelial cell monolayers: contribution of PECAM1. All the values are referred to measurements performed 1 h after albumin addition; results are expressed as albumin flux, pmol·cm^{-2}·s^{-1}. (A) Effect of undigested or F(ab')$_2$ fragment of the anti-PECAM M89D3 mAb. The anti-LFA3 mAb AICD58 was used as a control. (B) [^{125}I]Albumin transit through human PECAM1$^+$ transfectant (clone D12P and P-ECV) monolayers compared to mock transfected NIH/3T3 cells (clone E5) and the PECAM1$^-$ ECV304 human endothelial cell line. Effect of the F(ab')$_2$ fragment of anti-PECAM1 M89D3 mAb (5 μg/ml) on albumin flux, through clone D12P, clone P-ECV, ECV304 cell line or mock transfected E5. The anti-LFA3 mAb AICD58 was used as a control. Inset: surface labelling and immunoprecipitation of PECAM1 from E5 clone (mock), D12P clone, ECV304 cell line and P-ECV clone.

Table 1
Perturbation of endothelial barrier function in vivo by injection of anti-PECAM1 antibodies

Treatments[1]	Vascular leakage OD (540 nm)[2]	
	Liver	Kidney
Saline	324 ± 54	310 ± 50
Anti-PECAM1 mAb	1176 ± 103	1062 ± 53
Anti ICAM1 mAb	450 ± 34	378 ± 28
Anti L3T4 mAb	499 ± 13	322 ± 9

[1] Anesthetized mice ($n = 6$) were injected (in the vena cava or the renal artery) with either the anti-murine PECAM1 mAb (50 μg) or the anti-ICAM1 mAb (50 μg) or the anti-L3T4 mAb (50 μg) followed (after 15 min) by organ perfusion with Evans blue and washing with saline. Control is represented by dye-perfused, saline-washed organs. Maximum dye retention (dye perfusion without washing) yield an O.D. of 1056 ± 9.
[2] Liver and kidney were homogenized, centrifuged and the supernatants treated with 10% DOC. Samples were then run on a spectrofotometer at 540 nm wavelength. Results are the mean ± S.D. from 6 independent experiments.

comparable to that reported in the literature (about 0.2 pmol/h; ref [19]). Similar results were also obtained using microvascular endothelia isolated from human dermal vessels (not shown).

3.2. *Ectopically expressed PECAM1 contributes to monolayer integrity*

To confirm the function of PECAM1 in the maintenance of vascular barrier function, we generated cell clones stably expressing human PECAM1 from both murine fibroblasts (clone D12P) and from the human endothelial cell line ECV304 (clone P-ECV). Permeability to radiolabeled albumin was evaluated in D12P cells and compared to mock transfected NIH/3T3 murine fibroblasts or to the surface PECAM1$^-$ human endothelial cell line ECV304 and the transfected clone P-ECV obtained from the ECV304 cell line (Fig. 1B). PECAM1$^+$ stable transfectants (D12P and P-ECV) expressed considerable amounts of the molecule, a large fraction of which reaches the cell surface, as shown by immunoprecipitation after surface iodination (Fig. 1B, inset). Fig. 1B shows that albumin flux through PECAM1 transfected D12P and P-ECV was approximately 60% and 50% lower, respectively, than that observed in mock transfected NIH/3T3 or ECV304 cells. Moreover, the F(ab′)$_2$ fragment of the anti-PECAM1 mAb M89D3 increased the permeability of PECAM1 transfectants; by contrast, preincubation with the M89D3 mAb did not affect the transit of iodinated albumin in mock transfectants or in ECV304 cells (Fig. 1B), that do not express PECAM1 at the cell surface (Fig. 1B, inset). An anti-LFA3 mAb, used as a negative control, did not exert any effects on all the clones and cell lines tested (Fig. 1B).

3.3. *PECAM1 contribution to the maintenance of vascular barrier function is effective in vivo*

As PECAM1 contribution to endothelial integrity has been shown to be mainly effective during the formation of endothelial cell monolayers in vitro [9, 20], we addressed the question of whether a perturbation of vascular endothelial barrier function could be achieved in vivo by injection of the specific anti-murine PECAM1 mAb in mice. Leakage of Evans blue/albumin from liver and kidney vessels was evaluated by dye perfusion, according to Westergen et al. [21]. Injection of the anti-murine PECAM1 mAb (50 μg), followed by dye perfusion and washing with saline, caused an increase of dye retention by both liver and kidney, compared to saline or anti-murine ICAM1 or anti-L3T4 mAbs (an anti-murine LFA3 mAb was not available) (Table 1), possibly due to an increase in vascular permeability. Evans blue/albumin extravasation was also detected by immunoreactivity with an anti-albumin antibody (not shown). Similar results were obtained by the use of a vessel permeabilizing factor (tumor necrosis factor α) (not shown).

4. Discussion

In the present report we demonstrate that PECAM1, which has been described as a junctional structure expressed on EC monolayers [6–9], has a relevant function in the maintenance of vascular endothelium integrity both in vitro and in vivo.

In our experiments PECAM1 proved to be involved in endothelial barrier function. Indeed, transfection of PECAM1 cDNA into NIH/3T3 murine fibroblasts or into the ECV304 surface PECAM1$^-$ endothelial cell line, and subsequent expression of the protein at the cell surface, decreased the baseline permeability to macromolecules of transfectant monolayers compared to wild type or mock transfected cells. In all cases, the specific monoclonal antibody could selectively increase the albumin flux through the PECAM1$^+$ monolayers. The antibody effect was unlikely to be due to a signal delivered via PECAM1 clustering, as it was also detectable with the F(ab′)$_2$ (see section 3) and Fab′ (not shown) fragments. Moreover, we could not observe a 'capping' of the molecule by immunofluorescence nor major changes in morphology after EC incubation with the antibody (not shown). Thus, we favour the hypothesis of an interference of the antibody with homodimeric PECAM1–PECAM1 interactions in EC contacts.

These results indicate that PECAM1 contributes to the strengthening of cell–cell contacts whereby vascular endothelium can maintain its integrity and exert its functions. It has to be noted that D12P and P-ECV lines originated from two different cell types: fibroblasts and endothelial cells, the former reported to lack adherens or tight junctions, the latter equipped with other junctional molecules such as cadherins or integrins [1–4]. This might explain the more pronounced effect of the anti-PECAM1 antibody in the transfected fibroblasts, where the molecule plays indeed a mechanistic role in the strengthening of the intercellular contacts, as judged by the decreased levels of baseline permeability resulting from PECAM1 expression. Conversely, the other junctional molecules might provide P-ECV with additional tools for the maintenance of barrier integrity: this is also supported by the finding that co-incubation of EC with mAbs directed against known endothelial cadherins leads to an additional increase in albumin flux.

So far, it has been claimed that PECAM1 contribution to endothelial integrity is mainly confined to the formation of the endothelial cell monolayers in vitro, whereas it is not primarily involved in the perturbation of already established monolayers [9,20]. Thus, we addressed the question of whether a perturbation of vascular barrier function could be achieved in vivo by injection of the specific anti-PECAM1 antibody in mice. At variance with the above mentioned reports, all referred to in vitro experiments, we found that vascular endothelium be-

comes highly permeabile as a consequence of anti-PECAM1 mAb infusion, thus supporting the hypothesis that PECAM1-mediated cell–cell contacts are operating in vivo and contribute not only to the formation but also to the maintenance of endothelium integrity.

In conclusion, PECAM1 appears to be one of the molecules responsible for the endothelial continuity required for the maintenance of vascular barrier function: thus, we would imply that an abnormal or altered localization of PECAM1 along the endothelial cell membrane might contribute to the pathogenesis of both acute and chronic diseases involving vascular endothelium.

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