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Constitutive expression of E-and P-selectin cognate ligands in human endothelial cells

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ON human endothelial cells from umbilical cord (HUVEC) are present, in addition to E- and P-selectins, their cognate ligands. Differently from selectins, the ligand expression is constitutive and not modulated by interleukin-1β. Such ligands appear to be different from the ones present in promyelocytic cells in order to promote cell adhesion to immobilized selectins. The expression of selectin-ligands on HUVEC cells suggest that selectins can participate in endothelial signalling besides their role as adhesion molecules for circulating blood cells. However, despite their role in chemotaxis, selectins do not contribute to HUVEC tube formation in Matrigel.

Key words: adhesion molecules, selectin ligand, tube formation, endothelial adhesion, HUVEC

Introduction

The selectins initiate many critical interactions among blood cells and the vascular system. The recognition between E- and P-selectins expressed on activated endothelium and cognate ligands expressed on myeloid and lymphoid cells mediates the initial attachment of leukocytes to venular endothelial cells before their firm adhesion and diapedesis at sites of tissue injury and inflammation.[1] Besides their main role as adhesion molecules, selectins have been reported to participate in angiogenesis responses.[2] Soluble E-selectin has been demonstrated to promote human endothelial cell migration and to stimulate angiogenesis in the rat cornea.[3] In an *in vitro* model of angiogenesis, the formation of tube-like structures by bovine capillary endothelial cells was inhibited by the addition of an anti-E selectin antibody.[4] P-selectin, as well as E-selectin, recognizes sialylated glycans such as sialyl-Lewis X and sialyl-Lewis A containing molecules.[5] These molecules have also been implicated in capillary tube formation.[4] Consistently, we recently reported that soluble P-selectin can also promote human and bovine endothelial cell migration.[6] The expression of cognate ligands on endothelial cells is mandatory to support a role for selectins as molecules involved also in endothelial signalling and cross-talks. In this study we have assessed whether quiescent or interleukin-1 β (IL-1 β) primed cultured human endothelial cells possess molecules able to recognize E- and P-selectins and their effect in mediating endothelial functions such as adhesion and morphogenesis of capillary-like structures.

Materials and Methods

Cell lines and culture conditions

Human umbilical cord vein endothelial cells (HUVEC) were isolated with collagenase perfusion of term umbilical cords as previously described[7] and primary cultures were used in the experiments. HUVEC were grown in Medium 199 supplemented with 10% heat-inactivated fetal calf serum (65°C, 30 min). Undifferentiated human promyelocytic cell lines HL-60 and U937, obtained from the American Type Culture Collection (Rockville, MD), were mantained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum.

Reagents

Human recombinant basic fibroblast growth factor (bFGF) was purchased from Boehringer Mannheim (Germany). Human soluble E- and P-selectins were purchased from R&D System Europe Ltd, Abingdon, UK. The E-selectin–immunoglobulin fusion protein was kindly provided by Dr M. Bevilacqua. The following murine monoclonal antibodies (MoAb) were used: anti-human E-selectin, BBA 2 (R&D System Europe Ltd); anti-human P-selectin, AC1.2 and BMS126 (Becton Dickinson, San Jose, CA and Bender Med-System, Vienna, Austria, respectively); anti-Sialyl-Lewis X, CSLEX1 (Becton Dickinson). The second step antibody conjugates were: alkaline phosphatase goat anti-mouse total immunoglobulin (Ig) (Oncogene

Research Products, Cambridge, MA); alkaline-phosphatase conjugated goat anti-human IgG (Fc specific) (SIGMA Chemical Co., St Louis, MO).

Cell-ELISA assays

Confluent HUVEC (5×10^4 cells/well) stimulated or not with 100 U/ml of recombinant human IL-1 β (specific activity 1.3×10^7 U/mg; Janssen Biochimica, Beerse, Belgium) for 4 h at 37°C or HL-60 cells (3×10^5 cells/well; immobilized on 96-well plates coated with 0.1 mg/ml of poly-D-lysine, MW > 300 000; Sigma) were used in cell-ELISA experiments. Briefly, cells were washed in PBS and incubated for 1 h at 4°C in PBS containing 1% bovine serum albumin with primary MoAbs, the E-selectin-Ig fusion protein or the complex formed by soluble P-selectin and the corresponding non-neutralizing MoAb AC1.2. After washings, the appropriate alkaline-phosphatase conjugated goat anti-mouse total Ig or anti-human IgG were added to the wells for 45 min. Specific binding was calculated by subtracting the signal generated (conversion of phosphatase substrate) in the wells containing cells treated only with the appropriate alkaline-phosphatase conjugated goat anti-Ig. All samples were assayed in triplicate.

Cell adhesion assay

Cell adhesion assays were based on the procedure of Martens *et al.*,[8] with some modifications. Briefly, soluble E- and P-selectins were immobilized on 96-well plates in carbonate buffer $NaHCO_3$ Na_2 CO_3 0.05 M pH 9.6 (5 µg/ml overnight at 4°C and 10 µg/ml 1 h at room temperature for E- and P-selectins, respectively). The plates were then saturated with PBS containing 1% bovine serum albumin. HL-60, U937 and HUVEC cells (stimulated or not with 100 U/ml of recombinant human IL-1 β for 4 h at 37°C and then detached from culture plates by trypsinization) were added at 2×10^5 cells/well to the selectin-coated wells and incubated at room temperature for 45 min. Non-specific binding was determined in wells treated only with carbonate buffer. Wells were washed gently twice with PBS and the adherent cells were labelled with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The absorbance was read in a photometer Microplate 3550 at two wavelengths (690 nm and 570 nm as reference). In some experiments, HL-60 and U937 cells labelled with 2 µCi/ml [^{3}H]-methyl-thymidine (NEN-Du Pont DeNemours Italiana, Cologno Monzese (MI), Italy, 82.5 Ci/mmol) for 18 h, were used for the cell adhesion assays. ^{3}H radioactivity was determined by counting in a Wallac Betaplate™ liquid scintillation counter.

In vitro *angiogenesis model*

Morphogenetic experiments were performed according to the procedures of Wiedermann *et al.*[9] Briefly, a complete differentiation of HUVEC into capillary-like structures, was achieved by coating 24-well plates with 300 µl of Matrigel (Becton Dickinson, Bedford, MA, USA) per well, which was allowed to polymerize at 37°C for 30 min. For suboptimal stimulation of HUVEC differentiation, 300 µl per well of diluted Matrigel (1:2 with Medium 199) was kept at 4°C overnight for slow polymerization, followed by 37°C for 30 min before use. These suboptimal conditions were selected in order to better detect factors that promote tube-formation. HUVEC were suspended in Medium 199 + 10% FCS (10^5 cells per well), incubated for 30–40 min at 37°C with different concentrations of test substances and then plated on diluted Matrigel. Each condition was tested in triplicate wells. After 12–18 h of incubation at 37°C in 5% CO_2, the capillary-like structures were observed by inverted microscopy and quantitatively evaluated counting tubular formations $\geq$300 µm at 30× magnification and using an ocular grid.

Results

Cognate ligands for E- or P-selectins in HUVEC cells

To determine whether HUVEC cells express ligands for selectins, cell ELISA experiments were performed using the E-selectin–immunoglobulin fusion protein to recognize structures that bind E-selectin and a complex of soluble P-selectin with a corresponding non-neutralizing MoAb (AC1.2) to recognize ligands for P selectin. Moreover, MoAbs against sialyl-Lewis X, E-selectin and P-selectin were applied. The human promyelocitic cell line HL-60 immobilized on plate surface coated with poly-D-lysine, endowed with P- and E-selectin ligands,[10,11] was used as a positive control. As shown in Table 1, molecular structures able to bind both E- and P-selectin were found on cultured HUVEC cells. The MoAb CSLEX1 that recognizes sialyl-Lewis X,[12] a carbohydrate mojety bearing on ligands for selectins,[5] did not react above the background levels on HUVEC cells while was strongly recognized on HL-60. As expected, the reactivity of MoAb against E-selectin was detected only on HUVEC stimulated with IL-1 β while no reactivity for P-selectin was found. On the other hand, IL-1 β exposure did not affect the expression of E- or P-selectin cognate ligands.

Role of E- and P-selectin cognate ligands on cell adhesion mediated by immobilized selectins

The function of ligands for E- and P-selectins expressed on HUVEC was checked on adhesion assays to immobilized selectins in static conditions. As

Table 1. Detection of selectins and their cognate ligands on HUVEC and HL-60 cells through cell-ELISA assays

	HUVEC		HL-60
	Unstimulated	IL-1β-stimulated	
MoAb anti-sialyl-Lewis X (CSLEX 5μg/ml)	0 ± 0[a]	0 ± 0	0.673 ± 0.01
MoAb anti E-selectin (BBA2 10μg/ml)	0 ± 0	0.746 ± 0.04	0 ± 0
MoAb anti P-selectin (AC1.2 10μg/ml)	0 ± 0	0 ± 0	0 ± 0
P-selectin binding (sP-selectin 10μg/ml + MoAb AC1.2 10μg/ml)	0.533 ± 0.1	0.477 ± 0.1	0.445 ± 0.1
E-selectin binding (E-selectin-Ig fusion protein 50μg/ml)	0.113 ± 0.03	0.110 ± 0.02	0.126 ± 0.01

[a]Mean of absorbance (obtained subtracting the signal generated in the wells containing cells treated only with the appropriate alkaline-phosphatase conjugated goat anti-Ig) ± SD of three independent experiments.

shown in Table 2, HUVEC did not adhere to immobilized E- or P-selectin within 45 min of exposure time. Consistent with the lack of effect of IL-1 β on selectin-ligand expression, the cytokine treatment did not improve HUVEC adhesion. The human cell line HL-60 and U937 which express ligands for selectins adhered strongly to immobilized E- or P-selectins. This adhesion was selective and specific since it was blocked by the respective neutralizing MoAbs against E- (BBA2) or P-selectins (BMS 126). In fact, MoAb BBA2 (5 μg/ml) inhibited HL-60 or U937 adhesion to immobilized E-selectin of 62 ± 3.6% and 67 ± 8%, respectively (n = 3), while left inalterated the cell adhesion to P-selectin. Conversely, MoAb BMS126 (10 μg/ml) did not influence static adhesion to E-selectin but strongly blocked the HL-60 or U937 adhesion to P-selectin with inhibition of 92 ± 1%or 87 ± 4%, respectively (n = 3).

Role of E- and P-selectins in in vitro *assay for vascular morphogenesis*

We then assessed whether the presence of E- and P-selectin ligands on HUVEC cells could be instrumental to morphogenetic processes required in angiogenesis. The addition to Matrigel diluted 1:2 of increasing concentrations of the angiogenic factor bFGF, dose-dependently stimulated HUVEC to progress from small fragments of unconnected tubes or cell aggregates into capillary-like structures within 18 h (Figs 1 and 2). However, soluble E- or P-selectins (20–2000 ng/ml) did not elicit HUVEC differentiation in Matrigel diluted 1:2 (Fig. 2) although the same concentrations were efficient in promoting HUVEC chemotaxis.[3,6] In addition, soluble selectins or neutralizing MoAbs anti-E- or anti-P-selectin (BBA2 and BMS126 up to 20 μg/ml) did not modulate bFGF-induced tube-formation (data not shown).

Discussion

The role of selectins in angiogenesis[2] and in particular the effects of soluble E- and P-selectins on HUVEC migration reported by Koch and us,[3,6] suggested the presence of receptive molecules for selectins on endothelial cells themselves. A cell-ELISA assay based on direct binding of E- and P-selectins was used to answer this question and also to overcome the aspect

Table 2. HL-60, U937 and HUVEC cells adhesion on immobilized E- and P-selectins in static assays

Cells[a]	Assays[b]	Adhesion			
		E-selectin	Without E-selectin	P-selectin	Without P-selectin
HL-60	c.p.m.	39639 ± 6176[c]	387 ± 63	18887 ± 6176	148 ± 42
	o.d.	0.683 ± 0.01	0 ± 0	0.330 ± 0.03	0 ± 0
U937	c.p.m.	19124 ± 3907	357 ± 44	17078 ± 3870	436 ± 124
	o.d.	0.937 ± 0.1	0 ± 0	0.700 ± 0.08	0 ± 0
HUVEC	o.d.	0 ± 0	0 ± 0	0 ± 0	0 ± 0
IL-1β stimulated HUVEC	o.d.	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a]HL-60, U937, HUVEC and IL-1β-stimulated HUVEC cells (2×10^5 cells/well) were incubated for 1 h at R.T. in wells coated with immobilized E- and P-selectins or in wells treated only with carbonate buffer.
[b]The bound cells were expressed in c.p.m. in assays with radiolabelled cells or in o.d. in colorimetric assays.
[c]The results are reported as mean ± SEM of three independent experiments.

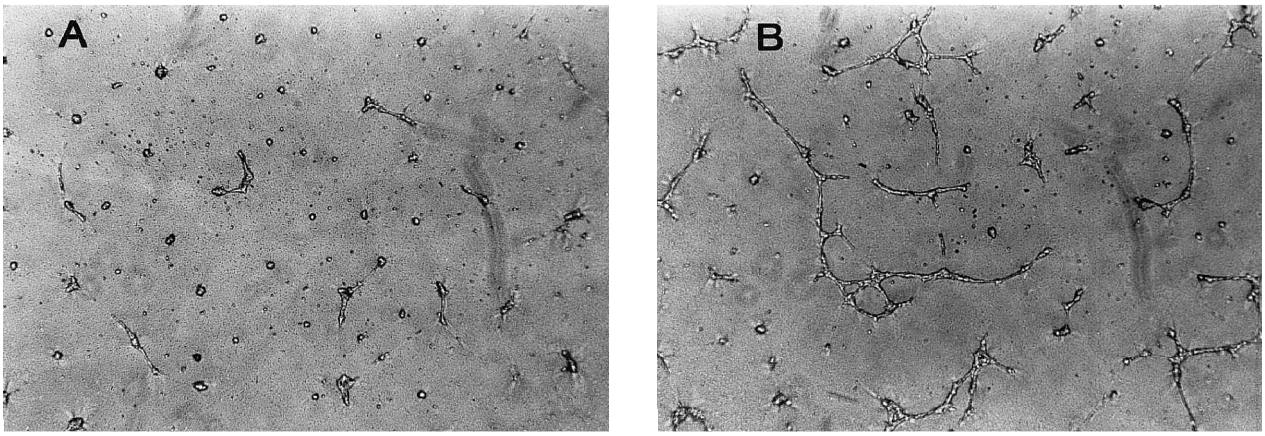

FIG. 1. Tube formation in Matrigel by HUVEC cells in the absence (A) or in the presence (B) of bFGF (20 ng/ml).

relative to the multiplicity of selectin ligands isolated until now in myeloid or cancer cells.[5,13–15] In this experimental condition we identified a constitutive presence of cognate ligands for E- and P-selectins on HUVEC cells. These structures could be responsible for transducing signalling inside the cells, since the endothelial responses to soluble form of selectins.[3,6] In addition, this ability was already proved for some E-selectin ligands, such as ESL-1, a variant of FGF receptor.[16] The presence of such signalling-receptor on HUVEC cells may suggest that they can be activated not only by soluble selectins, present in blood circulation following shedding of membrane bound selectins,[17] but also by the transmembrane selectins expressed in neighbouring activated endothelial cells or by the P-selectin present in platelets. Moreover they could be involved in activation of other endothelial functions during inflammatory process, besides their role in chemotaxis. In addition, the appearance of cellular function in response to such interactions is regulated by the presence of transmembrane or soluble selectins since expression of selectins arise following activation while selectin ligands are constitutively present on plasma membrane and are not modulated by IL-1 β stimulation. At present, we cannot exclude that the structure binding E- or P-selectins highlighted on endothelial cells in our experiments are the well-known L-selectin ligands. In fact it has been reported that an E-selectin-Ig chimera specifically stained HEV in mouse lymph nodes, as did an L-selectin-Ig chimera and precipitated GlyCAM-1 and CD34/Sgp90 although with less efficiency than L-selectin.[18] In any case, the finding of expression of cognate ligands for P-selectins is a novelty.

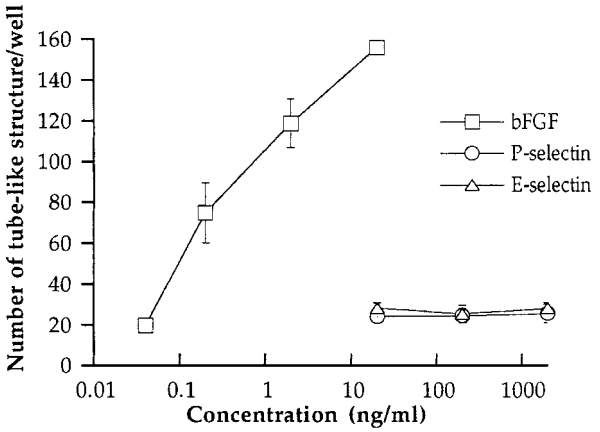

FIG. 2. Effect of bFGF and soluble E- and P-selectins on tube formation in Matrigel by HUVEC cells. The number of tube-like structures in unstimulated cells was 19 ± 2.

Since ligands for E- and P-selectins acts mainly as counter-receptor for adhesion molecules and mediate rolling and adhesion of inflammatory or cancer cells on endothelium we checked if the one expressed on HUVEC cells are able to mediate adherence to immobilized selectins through static adhesion assays. Unexpectedly, trypsinizated HUVEC cells were unable to adhere to E- or P-selectins even when stimulated with IL-1 β according to the no increased expression of the selectin ligands after cytokine treatment. Trypsinization was not a limiting step in adhesion since it did not alter the expression of selectin ligands. In fact, HUVEC cells exposed to trypsinization showed an immunoreactivity for the complex able to bind cognate ligands for P-selectins similar to the one observed in HL-60 in FACS analysis, although this methodology was less sensitive than cell-ELISA (our unpublished observation). There was a pharmacological and functional difference in mediating adhesion response between selectin cognate ligands expressed on HUVEC and myeloid or cancer cells. The absence of HUVEC adhesion to selectins could be linked to the dissimilarity in their molecular structures but also to the unfavourable setting for endothelial cells or to a low or inappropriate density of selectin ligands expression. However, the structural differences between cognate ligands for selectins on HUVEC and HL-60 cells were pointed out by the immunoreactivity for the MoAb anti-sialyl-Lewis X (CSLEX1). Specific carbohydrate structures can function as ligands for selectins, interacting, at least in part, with their lectin domains.[19–21] In general,

ligands for E- and P-selectins bear moieties of sialyl-Lewis X.[5] The antibody CSLEX1, which is able to inhibit the E- or P-selectin-mediated adhesion for non-lymphoid leukocytes and tumour cells of diverse origin[11,15] was strongly recognized on HL-60 cells but not on HUVEC cells. However, we cannot rule out that sialyl-Lewis X moieties are present on cognate ligands for selectins expressed on HUVEC cells since monoclonal antibodies anti-sialyl-Lewis X (FH-6 and KM-93) other than CSLEX1 were strongly recognized by HUVEC cells.[22]

It has been reported that selectins contribute to angiogenesis but their relevance in neovascular development has been debated. Besides the effect of E- and P-selectin in HUVEC chemotaxis,[3,6] we gathered evidence that they were not effective in switching on/off the programme of formation of capillary-like structures on Matrigel. This event is a defined process that does not require extensive proliferation and where only some aspects of endothelial cell differentiation are involved.[23] Therefore, we cannot exclude that in different experimental conditions (other extracellular matrix proteins or endothelial cells with different origins) selectins and their ligands could participate in the endothelial morphogenetic process. In addition, membrane selectins are not important in HUVEC capillary organization on Matrigel since neutralizing monoclonal antibodies directed against cell-surface E- or P-selectins did not inhibit basal or bFGF-induced tube-formation. This is in agreement with a recent paper describing that endothelial cells from E-selectin deficient mice form, without alteration, capillary-like structures on fibronectin, Matrigel and collagen gels *in vitro*.[24]

In conclusion, we have demonstrated the constitutive presence of E- and P-selectin cognate ligands on HUVEC cells that points out a new role(s) for this class of adhesion molecules as signalling factors for endothelial cells themselves.

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