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Divergent Fates of P- and E-Selectins After Their Expression on the Plasma Membrane

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P-selectin and E-selectin are related adhesion receptors for monocytes and neutrophils that are expressed by stimulated endothelial cells. P-selectin is stored in Weibel-Palade bodies, and it reaches the plasma membrane after exocytosis of these granules. E-selectin is not stored, and its synthesis is induced by cytokines. We studied the fate of the two proteins after their surface expression by following the intracellular routing of internalized antibodies to the selectins. By immunofluorescent staining, P-selectin antibody was first seen in endosomes, then in the Golgi region, and finally in Weibel-Palade bodies. In contrast, the E-selectin antibody was detected only in endosomes and lysosomes. Subcellular fractionation of cells after 4 h chase confirmed the localization of P-selectin antibody in storage granules and of the E-selectin antibody in lysosomes. In AtT-20 cells, a mouse pituitary cell line, transfected with P- or E-selectin, only P-selectin was delivered to the endogenous adrenocorticotrophic hormone storage granules after endocytosis. Deletion of the cytoplasmic domain abolished internalization. In summary, after a brief surface exposure, internalized E-selectin is degraded in the lysosomes, whereas P-selectin returns to the storage granules from where it can be reused.

INTRODUCTION

P- and E-selectin are adhesion molecules, sharing the same domain composition: a lectin domain, followed by an epidermal growth factor-like domain, several repeats similar to those found in complement binding proteins, a transmembrane region, and a short cytoplasmic tail (Bevilacqua *et al.*, 1989; Johnston *et al.*, 1989). P-selectin is localized in the membrane of α-granules of platelets and Weibel-Palade bodies of endothelial cells (Stenberg *et al.*, 1985; Berman *et al.*, 1986; Bonfanti *et al.*, 1989; McEver *et al.*, 1989). Both are storage granules that rapidly release their contents when the cells are activated. Fusion of the granule membrane with the plasma membrane leads to the expression of P-selectin on the cell surface where it mediates the adhesion of monocytes and neutrophils (Hattori *et al.*, 1989; Larsen *et al.*, 1989; Geng *et al.*, 1990; Hamburger and McEver, 1990). P-selectin also binds chronically stimulated $CD4^+$T cells, other subsets of leukocytes (Damle *et al.*, 1992; de-Bruijne-Admiraal *et al.*, 1992; Moore and Thompson, 1992), and some cancer cells (Aruffo *et al.*, 1991; Stone and Wagner, 1993). E-selectin is expressed in the endothelial cells by de novo synthesis only after they are stimulated with cytokines. E-selectin also promotes adhesion of neutrophils, monocytes, subsets of T-lymphocytes, and some cancer cells to the endothelium (Bevilacqua *et al.*, 1987, 1989; Rice and Bevilacqua, 1989; Picker *et al.*, 1991; Shimizu *et al.*, 1991).

The surface expression of the two proteins is likely to be very important as it allows interaction of the leukocytes with the endothelium. The migration of the leukocytes into tissues is the central event in the inflammatory response and is responsible for successful host response to tissue injury and infection. The initial contact of P-selectin with leukocytes is thought to induce them to roll, so that they can firmly attach through other receptors, allowing emigration. This was initially demonstrated in vitro, simulating the flow conditions of postcapillary venules (Springer, 1990; Lawrence and Springer, 1991). In vivo, rolling of leukocytes in the postcapillary venules is compromised in P-selectin–de-

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ficient mice (Mayadas, Johnson, Hynes, and Wagner, unpublished data). Likewise, it is also possible that E-selectin may be involved in leukocyte rolling and movement of leukocytes to the sites of inflammation (Bevilacqua *et al.*, 1987).

The surface expression of P-selectin is rapid, seen within 5 min of stimulation, but is transient, reaching near basal levels in 20–30 min (Hattori *et al.*, 1989). E-selectin induction on the other hand has a lag of 2–4 h and declines over the next 24 h (Bevilacqua *et al.*, 1987). Many membrane proteins, after their surface expression, are known to recycle after internalization (Ktistakis *et al.*, 1990; Vaux, 1992). The internalization signal for many has been characterized in detail. Its main feature is the presence of an aromatic residue in a turn in the cytoplasmic domain, which promotes clustering into coated pits (Collawn *et al.*, 1990; Vaux, 1992). The cytoplasmic domain of P-selectin also appears to be responsible for its targeting to the storage granules (Disdier *et al.*, 1992; Subramaniam, Koedam, and Wagner, unpublished data). The fate of the surface expressed P-selectin is unknown. Although internalization of P-selectin has been suggested (Hattori *et al.*, 1989), its final destination is not known.The cytoplasmic region of E-selectin shares features with other surface glycoproteins that are internalized (Ktistakis *et al.*, 1990). The presence of aromatic residues in the cytoplasmic regions of both proteins also makes endocytosis a possible pathway. In this study we have examined the fate of the P- and E-selectins after cell surface expression in endothelial cells and in heterologous cell lines expressing these proteins.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECS) were isolated and maintained as described (Mayadas and Wagner, 1989). AtT-20 cells (a mouse pituitary cell line) were maintained as described (Koedam *et al.*, 1992). Chinese hamster ovary (CHO) cells, transfected with P- or E-selectin cDNA, were a generous gift from Dianne Sako and Glenn Larsen, Genetics Institute, Cambridge, MA (Larsen *et al.*, 1992).

Constructs and Transfections

The AtT-20 cell line expressing wild-type P-selectin (WT) was described (Koedam *et al.*, 1992). A mutant from which the cytoplasmic tail was deleted was constructed as follows: a fragment of 0.7 kilobase from the P-selectin cDNA, encompassing the transmembrane and cytoplasmic domains and the 3′ untranslated region, was excised using *Xba* I and *Bam*HI. This fragment was subcloned into the polylinker of the replicative form of the phage M13mp18. Site-directed mutagenesis in single-stranded M13 was performed using the Amersham kit (Arlington Heights, IL). The synthetic oligonucleotide 5′GCTAAGAAAGCGTTAGAGACAAAAAGATG 3′ was used to change the phenylalanine residue at position 757 into a stopcodon. This mutation resulted in deletion of the last 32 amino acids (construct Δ CT). The nucleotide sequence of the mutated insert in M13 was confirmed using the Sequenase kit (United States Biochemical, Cleveland, OH). The insert was removed using *Xba* I and *Bam*HI and ligated to the pSP65 vector (Promega Biotec, Madison, WI) (cut with *Hpa* I and *Bam*HI) and the 5′ portion of the P-selectin (cut with *Hpa* I and *Xba* I). Resulting colonies were tested by restriction mapping. Finally the full-length mutated P-selectin cDNA was subcloned into the expression vector pCMV3 and transfected into the AtT-20 cells as described (Koedam *et al.*, 1992). Full-length cDNA encoding E-selectin was a kind gift from Michael Bevilacqua (Howard Hughes Medical Inst, La Jolla). It was excised from the pCDM7 vector using *Xho* I and subcloned into the pCMV3 vector by blunt end ligation. It was co-transfected into AtT-20 cells with pRSVneo using the calcium phosphate kit supplied by BRL (Gaitherburg, MD).

Immunofluorescence Staining

For double label immunofluorescent staining of HUVECS, cells were induced with 500 pg/ml recombinant interleukin-1β (IL-1) (obtained from Dr. C. Dinarello, New England Medical Center) for 4 h, fixed, and permeabilized (Wagner *et al.*, 1982). E-selectin was stained with the monoclonal antibody (mAb) HEL 3/8.1.3 obtained from Tim J. Ahern, Genetics Institute, Cambridge, MA at 1:250 dilution followed by rhodamine-conjugated goat antibody to mouse IgG (Organon Teknika Cappel, Malvern, PA) at 1:500 dilution. P-selectin was stained with an affinity purified polyclonal antibody (a generous gift from Drs. Bruce and Barbara Furie) at 1:25 dilution and vWf with a polyclonal antibody (American Bioproducts, NJ) at 1:100 dilution. This was followed by fluorescein isothiocyanate (FITC)-conjugated goat antibody to rabbit IgG (ICN Immuno Biologicals, Lisle, IL) at 1:100 dilution. All incubations were for 30 min at 37°C.

Endocytosis

HUVECS on coverslips were incubated with a nonblocking monoclonal anti-P-selectin antibody AC 1.2 (Larsen *et al.*, 1989) or its Fab fragments at 10–15 μg/ml in 5a McCoy's media with 1% fetal bovine serum (FBS) at 37°C for 10–15 min. The Fab fragments were made with papain digestion, modified from Porter (1959). The cells were then stimulated with 10^{-4} M histamine for 15 min in the presence of the antibody. The coverslips were washed to remove the unbound antibody and histamine and incubated in fresh 5a McCoy's media with 1% FBS before fixation and permeabilization. The internalized P-selectin antibody complex was stained with rhodamine-conjugated goat antibody to mouse IgG at 1:500 dilution or FITC-conjugated goat antibody to mouse Fab fragments (Organon Teknika Cappel). Ten milligrams per milliliter horseradish peroxidase (HRP), a fluid phase marker (Griffiths *et al.*, 1989), was added together with histamine and anti-P-selectin antibody. Coverslips were stained with polyclonal anti HRP antibody (Sigma, St. Louis, MO) at 1:100 dilution, followed by FITC-conjugated goat antibody to rabbit IgG at 1:100 dilution. To identify the Golgi apparatus, FITC-conjugated wheat germ agglutinin (WGA) (Vector Labs, Burlingame, CA) at 1:50 dilution was used. Control experiments were done with mAbs to von Willebrand factor (vWf) (Synbiotics, San Diego, CA) or vimentin (Sigma, St. Louis, MO).

To study endocytosis of E-selectin, cells were incubated for 4 h with 500 pg/ml IL-1 at 37°C. The coverslips were washed three times and incubated with 10 μg/ml monoclonal anti E-selectin antibody HEL 3/8.1.3 at 37°C for 15 min. The remaining procedure was the same as for P-selectin. Lysosomes were labeled using a mAb BB6 to lamp-1 (kindly provided by Dr. M. Fukuda, La Jolla, California) at 1:100 dilution. In this case the anti-E-selectin antibody used for endocytosis was polyclonal (kindly provided by Roy Lobb, Biogen, Cambridge, MA) at 1:100 dilution. Chloroquine (100 μg/ml) (Sigma) was added at the 4-h time point and kept on the cover slips for 18 h (Östman *et al.*, 1992).

To study the endocytosis of P-selectin, AtT-20 cells transfected with WT, and the ΔCT P-selectin cDNA were incubated with 10–15 μg/ml AC1.2 and 5 mM 8-Bromo-cAMP for 30 min at 37°C. The WT clones were labeled with polyclonal adrenocorticotrophic hormone (ACTH) antibody (a gift from the National Hormone and Pituitary Program, University of Maryland, School of Medicine, College Park, MD) at 1:500 followed by FITC-conjugated goat antibody to rabbit IgG at 1:250 dilution. In case of ΔCT clones, staining was enhanced

by using a second rhodamine-conjugated rabbit antibody to the goat IgG. AtT-20 cells transfected with E-selectin cDNA were incubated with monoclonal anti E-selectin antibodies HEL 3/8.1.3 or BB11 (a generous gift from Roy Lobb, Biogen, Cambridge, MA) in Dulbecco's minimal essential medium with 1% FBS for 30 min and were further treated as for P-selectin. Nontransfected CHO cells and CHO cells transfected with P- or E-selectin cDNA were incubated with P- or E-selectin antibodies for 45 min at 4°C. Coverslips were washed, and endocytosis of the antibody antigen complex was observed by immunofluorescence staining. The coverslips were double labeled with a polyclonal 1gp120 antibody (a generous gift from Dr. Ira Mellman, Yale University, New Haven, CT) at 1:100 dilution followed by FITC-conjugated goat antibody to rabbit IgG at 1:100 dilution.

Fractionation of Endothelial Cells After Endocytosis

Endocytosis of P-selectin—bound antibody was studied as follows: endothelial cells in two 145-cm^2 dishes were incubated at 37°C with 4 ml of 5a McCoy's media 1% FBS, 1 μg/ml ^{125}I-labeled AC1.2, and 10^{-4} M histamine for 15 min. AC 1.2 was omitted in the two control dishes. Iodination was performed using Enzymobead radioiodination reagent (Bio-Rad Laboratories) (1 ng = 2522 cpm). The cells were washed and incubated for 4 h at 37°C. The cells were moved to 4°C and were subjected to an acid wash with 0.2 M acetic acid 0.5 M NaCl pH 2.4 for 5 min (Hopkins and Trowbridge, 1983). They were then washed three times. They were scraped in 5 ml ice-cold homogenization buffer (20 mM tris(hydroxymethyl)aminomethane, pH 7.2, 0.2 M sucrose, 1 mM EDTA), collected in a 15-ml tube, and centrifuged at 1500 rpm for 6 min. The pellet was resuspended in 1 ml homogenization buffer. In the control dishes, the iodinated antibody was added only at this juncture. The cells were broken by 50 strokes in a dounce homogenizer (size 19, Kontes Glass, Vineland, NJ) at 4°C, and the nuclei and cellular debris removed by centrifugation at 600 $\times$ g for 10 min. The postnuclear supernatant was layered gently on 8.5 ml, 35% Percoll and 0.25 M sucrose, pH 7.2, in 10 ml Oak Ridge tubes (Nalge Co, Rochester, NY) and then centrifuged for 60 min at 40 000 $\times$ g in a Sorvall RC-5B centrifuge (SM24 rotor). Twelve 800-μl fractions were collected from the top, and 1 mM phenylmethylsulfonyl fluoride and 12.5 μg/ml leupeptin were added. The fractions were freeze thawed before vWf determination by enzyme-linked immunoadsorbent assay (Koedam *et al.*, 1992). The lysosomal marker N-acetyl-β-glucosaminidase was assayed by the release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminidase (Sellinger *et al.*, 1960). Likewise for E-selectin four 145-cm^2 dishes were induced with IL-1 for 4 h, washed with ice-cold media, and incubated for 1 h at 4°C with 4 ml media/dish and 1 μg/ml ^{125}I-labeled HEL 3/8.1.3 (1 ng = 2833 CPM). Dishes were washed, and two dishes were transferred to 37°C for 4 h and two were left at 4°C. The rest of the procedure was the same as for P-selectin.

RESULTS

Distribution of P-Selectin and E-Selectin in IL-1–stimulated Endothelial Cells

Cytokine-stimulated endothelial cells express both P- and E-selectin. To study their relative distribution, endothelial cells were incubated with IL-1 for 4 h and then fixed, permeabilized, and stained both with monoclonal anti-E-selectin and polyclonal anti-P-selectin antibodies. E-selectin was visualized in heterogenous vesicles and on the cell surface (Figure 1a). P-selectin was seen exclusively in elongated storage granules, the so called Weibel-Palade bodies (Figure 1b). The two proteins did not colocalize. E-selectin was not stored in the Weibel-Palade bodies, as was also demonstrated by its distinct distribution from vWf, a soluble protein of these storage granules (Figure 1, c and d).

Endocytosis of P-Selectin Evaluated by Fluorescence Microscopy

Endothelial cells incubated with monoclonal anti P-selectin antibody were stimulated with histamine for 15

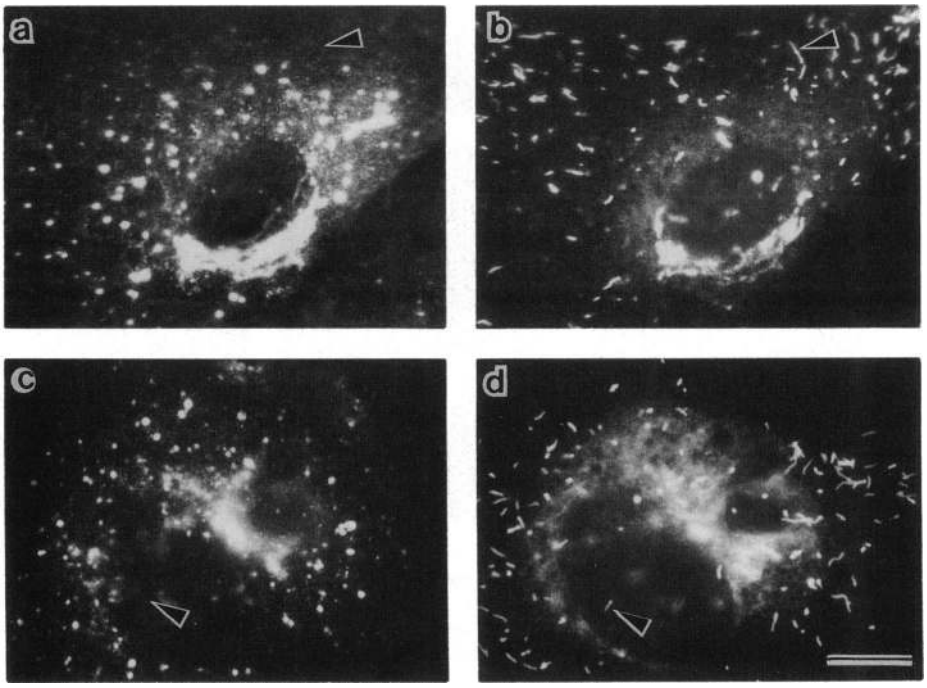

Figure 1. Distribution of P- and E-selectin in IL-1–treated endothelial cells. Human umbilical vein endothelial cells treated with 500 pg/ml IL-1 for 4 h were stained by double-label immunofluorescence. An identical field is shown in a and b and in c and d. The antibodies used are as follows: mAb to E-selectin (a), polyclonal antibody to P-selectin (b), mAb to E-selectin (c), and polyclonal antibody to vWf (d). Arrows point to P-selectin and vWf in elongated storage granules called Weibel-Palade bodies. E-selectin is not found in these granules. Bar, 10 μm.

min, and the P-selectin bound antibody was followed for 18 h by immunofluorescent staining. The P-selectin bound antibody was seen in a dotted pattern at the periphery of the cell within 10–30 min (Figure 2a). These were intracellular vesicles as they could not be seen in nonpermeabilized cells. To determine whether the vesicles were endosomes, the fluid-phase marker HRP, known to be taken up by endosomes (Griffiths *et al.*, 1989), was added during the histamine stimulation. Some of the internalized P-selectin indeed colocalized with HRP in the endosomes. After 1 h the internalized P-selectin was seen mostly in the perinuclear region (Figure 2b) where it colocalized with a Golgi apparatus marker, WGA (Wong *et al.*, 1992). Soon after, small budding Weibel-Palade body-like structures were seen originating from this region and spreading all over the cytoplasm by 4 h (Figure 2c). The process of transfer of the internalized antibody to the Golgi region and then to Weibel-Palade body-like structures was remarkably synchronized among the cells of a given culture. That these elongated organelles containing the endocytosed P-selectin–bound antibody were indeed Weibel-Palade bodies was shown by double label staining for vWf (Figure 3, a and b). The internalized antibody to P-selectin was found only in a subset of Weibel-Palade bodies (varied from 30–70%), presumably those that formed at the time that the internalized P-selectin reached the Golgi apparatus. The antibody to P-selectin in the Weibel-Palade bodies was long lived as it could be visualized even 18 h after endocytosis (Figure 2d). Similar results were observed with Fab fragments and another monoclonal anti P-selectin antibody 1.18 (gift from Dr. Jan Sixma, University of Utrecht, The Netherlands). The Weibel-Palade bodies containing the internalized antibody could be released again, because their numbers were greatly reduced upon restimulation with histamine. One of our concerns was the possibility that the antibody may dissociate from the antigen in the acidic milieu of the endosome or the trans-Golgi apparatus. To test the pH dependence of the antibody-antigen dis-

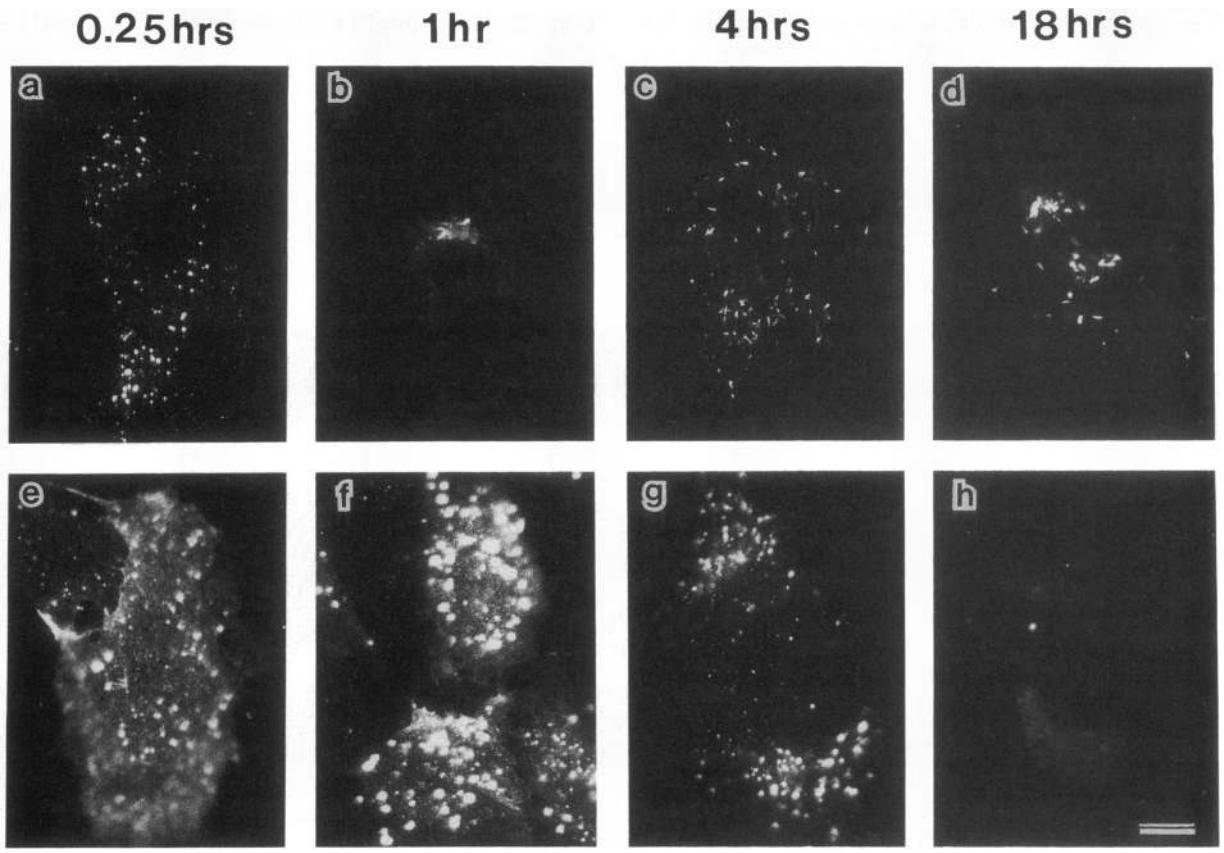

Figure 2. Endocytosis of P- or E-selectin–bound antibody, visualized by immunofluorescent staining. (a–d) Endothelial cells incubated with monoclonal P-selectin antibody at 37°C were stimulated with histamine for 15 min. The cells were fixed at the indicated time points, permeabilized, and stained with rhodamine-conjugated anti-mouse IgG. P-selectin–bound antibody is seen in endosomes (a), Golgi region (b), and Weibel-Palade bodies (c and d). (e and f) Endothelial cells stimulated for 4 h with IL-1 were incubated with monoclonal E-selectin antibody. The E-selectin–bound antibody is seen in endosomes (e) and (f) and lysosomes (g). By 18 h only trace amounts remain (h). The final destination and the intracellular routing of the two antibodies are clearly different. Bar, 10 μm.

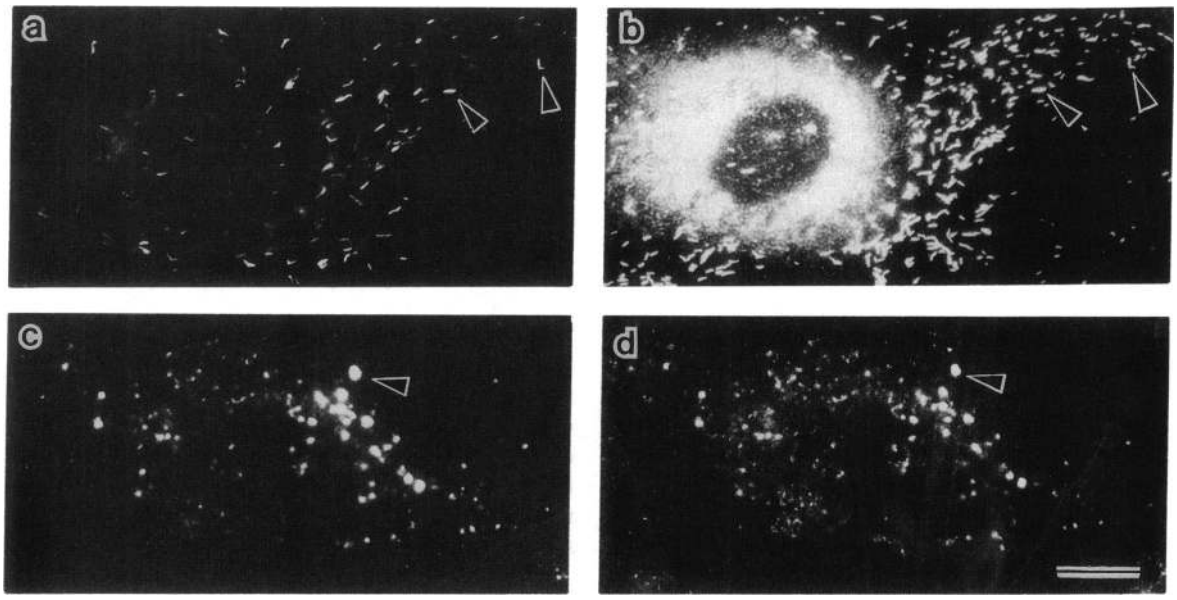

Figure 3. Colocalization of P- and E-selectin–bound antibodies with other cellular components 4 h after endocytosis in the endothelial cells. Arrows show P-selectin–bound antibody (a) that colocalizes with vWf, visualized with a polyclonal antibody in the Weibel-Palade bodies (b). In contrast, internalized E-selectin–bound polyclonal antibody (c) colocalizes with lamp-1 in the lysosomes (d). Bar, 10 μm.

sociation, the anti-P-selectin antibody was bound to CHO cells expressing P-selectin on their surface at 4°C. The cells were then washed and incubated for 30 min at pH 7.2 or 5.5. The amount of antibody that remained bound, determined by fluorimetry, was similar under both conditions. We have also checked that the endothelial cells did not become generally permeable to antibodies by incubating the live cells in our experimental conditions with mAbs to vimentin. Staining of the fixed permeabilized cells did not reveal the intermediate filaments pattern. Similar experiments were performed also with a monoclonal anti-vWf antibody and no recycling of vWf to the granules was seen.

Endocytosis of E-Selectin Evaluated by Fluorescent Microscopy

In parallel experiments to those described above, we studied the fate of E-selectin after surface expression. Endothelial cells stimulated with IL-1 for 4 h were labeled with an anti-E-selectin mAb HEL 3/8.1.3 for 15 min. The E-selectin–bound antibody was internalized to endosomes and heterogeneous vesicles, reminiscent of the degradative pathway (Figure 2, e–g). At 4 h E-selectin–bound antibody was found in the lysosomes as demonstrated by double label staining with anti-lysosomal membrane protein (lamp-1) antibody (Figure 3, c and d). Most of the E-selectin bound antibody was degraded by 18 h (Figure 2h). When the cells were treated with chloroquine, the antibody persisted after 18 h in large heterogenous vacuoles, because chloroquine inhibited the capacity of the lysosomes to degrade the protein (Östman *et al.*, 1992). Similar to the antibody, E-selectin was also degraded, because 18 h after the IL-1 induction the cells contained only trace amounts of E-selectin (Bevilacqua *et al.*, 1989).

Intracellular Distribution of Internalized P-Selectin Determined by Cell Fractionation

To further investigate the cellular distribution of the endocytosed P-selectin antibody, we performed cell fractionation. Endothelial cells were incubated with ^{125}I-anti-P-selectin antibody and histamine for 15 min and washed. Four hours later the surface-bound antibody was removed by an acid wash. Cells were homogenized and fractionated in a Percoll gradient. Twelve fractions were collected from the top of the gradient. Most of the antibody was found in the dense fractions 8–11 (Figure 4). Weibel-Palade bodies containing the large vWf multimers are located in these fractions (Ewenstein *et al.*, 1987; Vischer and Wagner, 1993). The peak of the P-selectin antibody was in fraction 8, containing the lighter Weibel-Palade bodies, that likely represent the newly formed and less well condensed organelles (Matsuda and Sugiura, 1970). We have also observed that newly synthesized vWf is first incorporated into the lighter Weibel-Palade bodies fractions (Subramaniam, Koedam, and Wagner, unpublished data). The lysosomal peak was recovered in fractions 3 to 6 and contained little of the internalized antibody (Figure 4). The other subcellular organelles such as the endoplasmic reticulum, the Golgi apparatus, the mitochondria, and the plasma membrane are all found in lighter fractions than the

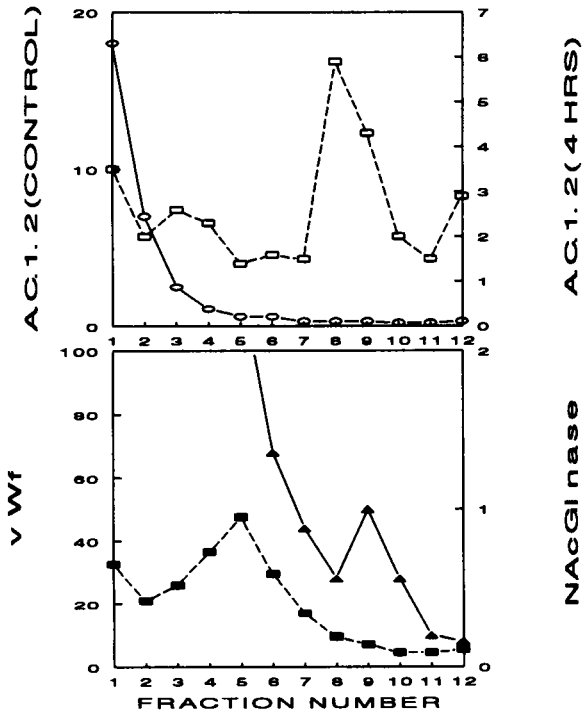

Figure 4. Subcellular distribution of internalized P-selectin antibody in endothelial cells determined by cell fractionation. Endothelial cells that had internalized ^{125}I-P-selectin antibody AC1.2 at 37°C for 4 h were homogenized and overlayed on a 35% Percoll gradient in 0.25 M sucrose. Twelve 800-μl fractions were collected from the top (fraction 1 is the least dense), and percentage of total internalized counts was determined for each fraction. ^{125}I-antibody was recovered in two peaks (□). The major peak at high density (fractions 8–11) is the granular peak containing vWf (▲, ng/ml). The lysosomal peak (■, OD at 420) was determined by the marker N-acetyl-β-glucosaminidase activity. The control, in which ^{125}I-antibody was added only during homogenization, is shown by open circles.

Weibel-Palade bodies (Vischer and Wagner, 1993). Endosomes are known to cosediment with the membrane fractions (Marsh *et al.*, 1987). The following control experiments were performed to rule out the possibility of "leaky" Weibel-Palade bodies accessible to exogenous antibody. Here the ^{125}I-labeled AC1.2 (directed to a part of P-selectin present in the lumen of the Weibel-Palade bodies) was added during homogenization. In this case, no radioactivity was found in fractions 8 to 11 (Figure 4). The absence of label in the Weibel-Palade body-containing fractions confirmed that the organelles were intact, and therefore the antibody could arrive into the granular fraction only through endocytosis.

Intracellular Distribution of Internalized E-Selectin Determined by Cell Fractionation

Endothelial cells were incubated with IL-1 at 37°C for 4 h to induce E-selectin expression. Surface E-selectin was labeled with anti-E-selectin antibody for 1 h at 4°C. The unbound antibody was washed off, and after 4 h of incubation at 37°C, cells were fractionated as described above. The peak of radioactivity corresponding to the internalized antibody was in fractions 3 to 6 coinciding with the lysosomal peak (Figure 5). The cells that were left at 4°C throughout the experiment showed little internalization as 80–90% of the antibody remained accessible to an acid wash (Figure 5).

Endocytosis of P-Selectin and E-Selectin Expressed in Heterologous Cell Lines

AtT-20, a cell line derived from a mouse anterior pituitary tumor, was used to study the endocytosis of the wild-type P-selectin and its tailless mutant (ΔCT). This cell line was chosen because it has a regulated pathway of secretion. Although transfected P-selectin is sorted to the endogenous ACTH granules, the ΔCT mutant is not stored and is seen on the cell surface (Disdier *et al.*, 1992; Koedam *et al.*, 1992). The endocytosis of the wild-type P-selectin and the ΔCT mutant was studied by stimulating the cells with the secretagogue 8-Bromo

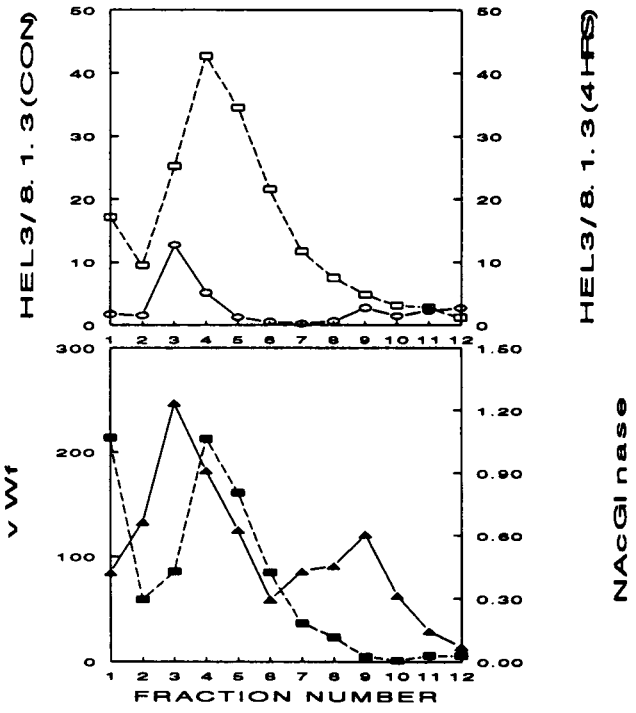

Figure 5. Subcellular distribution of internalized E-selectin antibody in endothelial cells determined by cell fractionation. ^{125}I-E-selectin antibody HEL3/8.1.3 was allowed to internalize for 4 h at 37°C in IL-1–treated endothelial cells. Cells were then fractionated as in Figure 4. ^{125}I-HEL 3/8.1.3 (□, cpm × 10^3) was recovered in the fractions containing N-acetyl-β-glucosaminidase (■, OD at 420). This peak is distinct from the granular peak seen with vWf (▲, ng/ml). The control cells that were kept at 4°C throughout the experiment showed little endocytosis (O, cpm × 10^3) as most of the antibody was removed by acid wash.

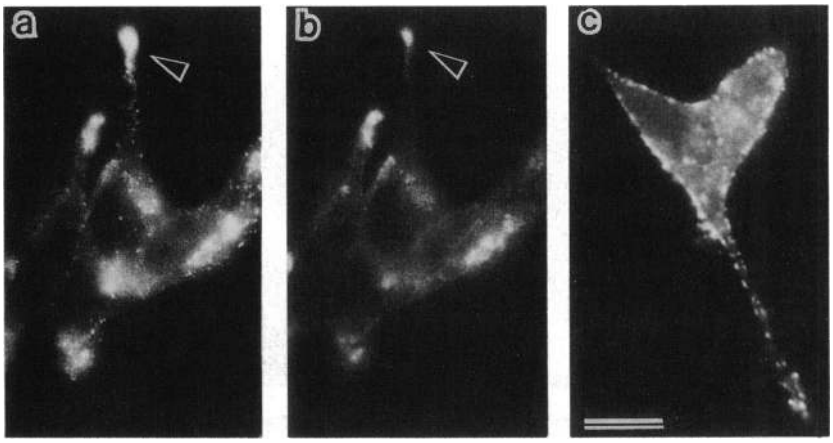

Figure 6. Endocytosis of the P-selectin—bound antibody in AtT-20 cells. AtT-20 cells transfected with wild-type (a and b) or ΔCT mutant (c) cDNA were incubated with monoclonal P-selectin antibody and 8-Br-Cyclic AMP at 37°C for 30 min. At 4 h cells were fixed, permeabilized, and stained for ACTH (a) or for mouse IgG (b). Arrows point to the internalized antibody seen in storage granules at the tip of the cells expressing wild-type P-selectin (b). The ΔCT mutant-bound antibody is seen on the surface (c). Bar, 10 μm.

cyclic AMP in the presence of the a P-selectin antibody. Internalization was followed by immunofluorescent staining as described for the endothelial cells. The wild-type P-selectin was internalized to the storage granules (Figure 6) containing ACTH (Figure 6a), whereas the ΔCT mutant was not internalized and remained on the surface (Figure 6c). Similar to the endothelial cells, E-selectin was internalized but did not go to the granules (Figure 7, a and b).

We have also followed endocytosis of P- and E-selectin in CHO cells transfected with their respective cDNA. The cells were incubated with mAbs for 45 min at 4°C and then transferred to 37°C. At 0 min surface staining of both antibodies were seen (Figure 8, a and c). Subsequently, antibodies were internalized in both cell types (Figure 8, b and d). Because CHO cells do not have storage granules (D. Wagner *et al.*, 1991), antibodies bound to either selectin were seen in lysosomes as confirmed by double staining with antibody to 1gp120. In nontransfected CHO cells treated in the same way no internalized antibody could be detected.

DISCUSSION

Endothelial cells synthesize two adhesion receptors of the selectin family. Although they are very similar in domain composition and have overlapping ligand specificities (Lasky, 1992), the stimuli that induce their surface expression are very different. P-selectin is a component of storage granules (Sternberg *et al.*, 1985; Berman *et al.*, 1986; Bonfanti *et al.*, 1989; McEver *et al.*, 1989) and is translocated to the plasma membrane after treatment of the endothelial cells with agents that cause vWf release, such as thrombin, histamine, complement components C5b-9, vascular permeability factor, and fibrin (Wagner, 1990). In contrast, E-selectin is not part of the Weibel-Palade body (Figure 1); it is synthesized de novo by endothelial cells stimulated with cytokines or endotoxin and is delivered to the plasma membrane directly after synthesis (Bevilacqua *et al.*, 1987, 1989). In this study we have compared the intracellular routing and fate of the two selectins after their surface expression.

After P-selectin is internalized, it is found in endosomes from where it appears to be transported to the Golgi region and finally to the Weibel-Palade bodies (Figure 2). The intracellular movement of P-selectin, tagged with an antibody, is synchronized from cell to cell. The majority appears in the Golgi region 60–90 min after P-selectin's surface exposure. Subsequently, the first Weibel-Palade bodies containing the endocy-

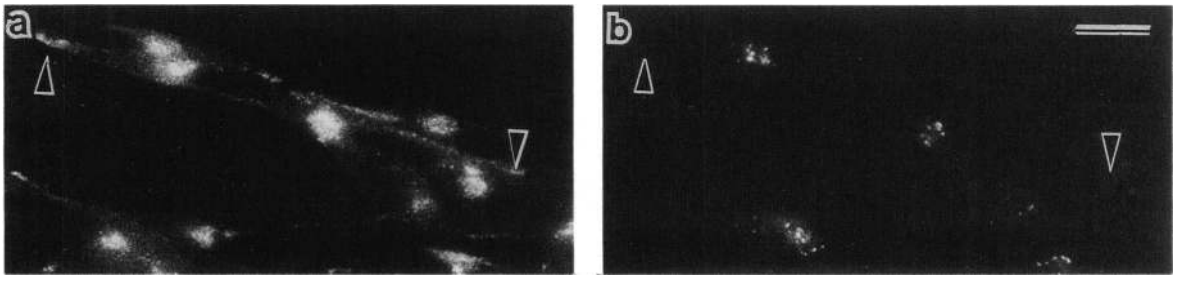

Figure 7. Endocytosis of E-selectin–bound antibody in AtT-20 cells. AtT-20 cells transfected with E-selectin cDNA were incubated with E-selectin mAbs BB11 at 37°C for 30 min. The antibody was washed off, and cells were further incubated in fresh media for 4 h. Coverslips were fixed, permeabilized, and double stained with rhodamine-labeled anti-mouse IgG (b) and with polyclonal antibody to ACTH (a). The internalized E-selectin–bound antibody is not seen in the storage granules at the tip of the cells (arrows) but rather in large vesicles located close to the nucleus. Bar, 10 μm.

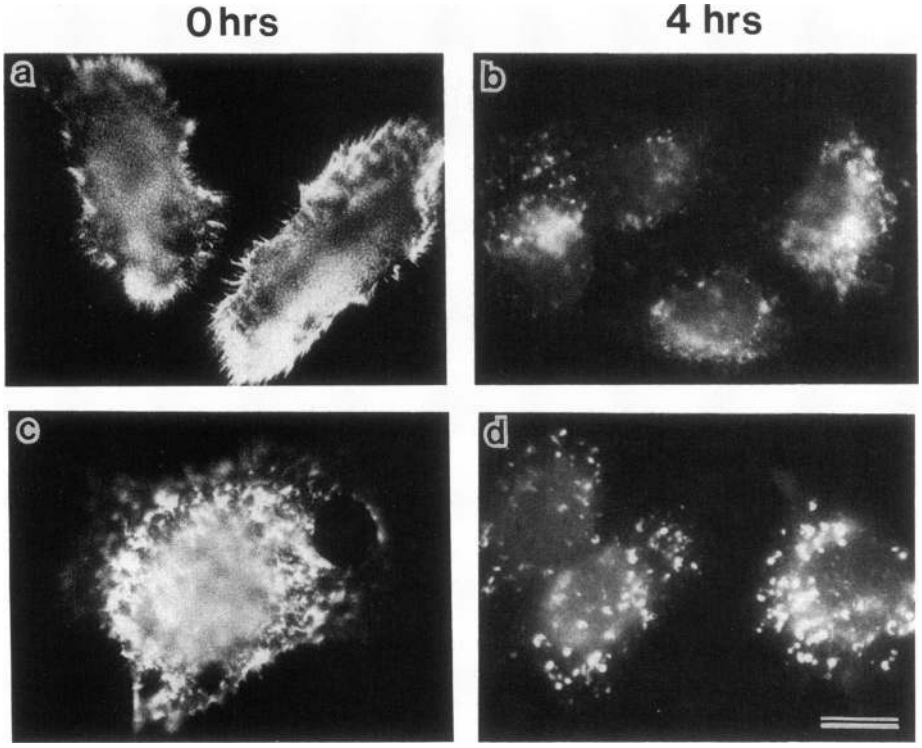

Figure 8. Internalization of P- or E-selectin antibody in CHO cells. CHO cells expressing P-selectin (a and b) or E-selectin (c and d) were incubated with the respective P- or E-selectin antibodies for 45 min at 4°C. They were washed and transferred to 37°C for 4 h. Cells were fixed, permeabilized, and stained with rhodamine-conjugated anti-mouse antibody. At 0 time both cell lines show surface staining (a and c), and at 4 h the antibody is seen in similar intracellular organelles (b and d). Bar, 10 μm.

tosed antibody appear budding from the Golgi region, and the free organelles then slowly distribute throughout the cytoplasm over a period of hours. The majority of the antibody molecules or Fab fragments do not dissociate from the P-selectin molecules to which they initially bound, because in vitro this antibody-antigen interaction persists even at low pH conditions. Cell fractionation of endothelial cells 4 h after endocytosis of the iodinated anti-P-selectin antibody shows that the antibody is found in the less dense subset of Weibel-Palade bodies (Figure 4). This pool of Weibel-Palade bodies likely represents the newly formed organelles whose density, similar to other known immature storage granules, may be lower (Matsuda and Sugiura, 1970; Tooze *et al.*, 1991; Subramaniam, Koedam, and Wagner, unpublished data).

The other proteins of storage granules whose fate after exocytosis was reported are glycoprotein III and dopamine β hydroxylase from chromaffin granules (Suchard *et al.*, 1981; Patzak and Winkler, 1986). In immunolabeling studies similar to ours, glycoprotein III was shown to return close to the Golgi region and at 6 h from endocytosis was found in organelles morphologically identical to chromaffin granules and in lysosomal structures (Patzak and Winkler, 1986). Synaptic vesicle membrane proteins also recycle back after endocytosis (Sudhof and Jahn, 1991). We have not observed significant amounts of internalized P-selectin in lysosomes (Figure 4), and the majority of P-selectin antibody-containing organelles also contained vWf (Figure 3). This indicates that the recycling of P-selectin to Weibel-Palade bodies is a very efficient process. We did not observe recycling of released surface-associated vWf (Sporn *et al.*, 1986) using an anti-vWf antibody. The surface-bound vWf therefore does not appear to be bound to P-selectin. This is in agreement with our unpublished data that P-selectin and vWf do not interact in vitro.

Many of the transmembrane proteins after endocytosis recycle through the Golgi apparatus. For example, the cation-independent mannose 6-phosphate receptor and the low-density lipoprotein receptor are transported to the Golgi apparatus with $t1/2 = 2–3$ h. Because the half-life of the proteins is >20 h, this relatively rapid recycling indicates that the receptors can return to the Golgi several times in their life span (Green and Kelly, 1992).

The signal on the P-selectin molecule that allows it to recycle from the cell surface to the storage granules is likely to be recognized by other cells with a regulated pathway of secretion, because the pituitary cell line AtT-20, transfected with P-selectin cDNA, internalized antibody bound P-selectin to the endogenous storage granules (Figure 6). In contrast, internalized E-selectin is found in lysosomal structures both in the endothelial cells (also recently reported by von Asmuth *et al.*, 1992) and in AtT-20 cells expressing this protein (Figures 3, 5, and 7). Whereas

in the storage granules P-selectin is protected from degradation, the internalized E-selectin in lysosomes is short lived (Figure 2).

The heterologous expression of P-selectin in AtT-20 cells has allowed us to show that the internalization signal of P-selectin is in the cytoplasmic domain of the protein (Figure 6). The targeting signal directing P-selectin to the storage compartment has also been shown to be part of the cytoplasmic domain (Disdier *et al.*, 1992; Koedam and Wagner, unpublished data). Whether the recognition sequences responsible for targeting of P-selectin to storage granules are similar to or distinct from those required for internalization is not known. The cytoplasmic domains of various receptors contain signals for functions such as targeting to the basolateral surface, sorting to the lysosomes, and internalization. These signals may be distinct (Goldstein *et al.*, 1985; Lobel *et al.*, 1989; Yakode *et al.*, 1992) or collinear (Harter and Mellman, 1992; Matter *et al.*, 1992). The signals in the cytoplasmic domain for internalization of various proteins are aromatic amino acids placed in a context of a tight reverse turn (Collawn *et al.*, 1990; Vaux, 1992). The cytoplasmic region of P-selectin comprises a tyrosine and three phenylalanine. Interestingly, the phenylalanine at position 785 is conserved as a tyrosine in the cDNA of the mouse P-selectin (Weller *et al.*, 1992). These aromatic amino acids may allow for interactions with adaptins or other cytoplasmic components mediating internalization. We have also shown that E-selectin is endocytosed and transported to lysosomes. In contrast to P-selectin, we did not detect E-selectin in the Golgi region after internalization. It is likely that E-selectin also carries an internalization signal in its cytoplasmic domain. This domain has two tyrosines (Bevilacqua *et al.*, 1989) surrounded by amino acids frequently found in turns that favor interaction with the adaptins (Ktistakis *et al.*, 1990). We do not know if the endocytosis of P- or E-selectin occurs through clathrin-coated pits. In endothelial cells, the amount of surface P-selectin decreases to near basal levels in 20–30 min after exocytosis (Hattori *et al.*, 1989), whereas surface expression of E-selectin diminishes over 24 h (Bevilacqua *et al.*, 1987). The difference in the length of surface expression of the two receptors could be because of differences in their rate of endocytosis or in their mode of delivery to the cell surface. They could be internalized at similar rates with the longer expression of E-selectin being because of recycling between endosomes and the surface. Alternatively, new E-selectin molecules may be deposited on the membrane over many hours, whereas P-selectin molecules are likely to be delivered to the surface all at once with the endocytosed P-selectin being retained in the storage granules. Naturally occurring mutations in the cytoplasmic domains of the selectin molecules could affect both intracellular targeting efficiency and the internalization of the surface expressed molecules. This could lead to an increased expression of the selectins on the plasma membrane resulting in excessive recruitment of the ligand-bearing leukocytes, leading to chronic inflammation.

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REFERENCES

Aruffo, A., Kolanus, W., Walz, G., Fredman, P., and Seed, B. (1991). CD62/P-selectin recognition of myeloid and tumor cell sulfatides. Cell *67*, 35–44.

Berman, C.L., Yeo, E.L., Wencel-Drake, J.D., Furie, B.C., Ginsberg, M.H., and Furie, B. (1986). A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. J. Clin. Invest. *78*, 130–137.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S., and Gimbrone, M.A., Jr. (1987). Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA *84*, 9238–9242.

Bevilacqua, M.P., Stengelin, S., Gimbrone, M.A., Jr., and Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science *243*, 1160–1165.

Bonfanti, R., Furie, B.C., Furie, B., and Wagner, D.D. (1989). PADGEM (GMP 140) is a component of Weibel-Palade bodies of human endothelial cells. Blood *73*, 1109–1112.

Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, V., Jing, S., Trowbridge, I.S., and Tainer, J.A. (1990). Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytes. Cell *63*, 1061–1072.

Damle, N.K., Klussman, K., Dietsch, M.T., Mohagheghpour, N., and Aruffo, A. (1992). GMP-140 (P-selectin/CD62) binds to chronically stimulated but not resting $CD4^+$ T lymphocytes and regulates their production of proinflammatory cytokines. Eur. J. Immunol. *22*, 1789–1793.

de-Bruijne-Admiraal, L.G., Modderman, P.W., Von-dem-Borne, A.E., and Sonnenberg, A. (1992). P-selectin mediates Ca(2+) dependent adhesion of activated platelets to many different leukocytes: detection by flow cytometry. Blood *80*, 134–142.

Disdier, M., Morrissey, J.H., Fugate, R.D., Bainton, D.F., and McEver, R.P. (1992). Cytoplasmic domain of P-selectin (CD62) contains the signal for sorting into the regulated secretory pathway. Mol. Biol. Cell *3*, 309–321.

Ewenstein, B.M., Warhol, M.J., Handin, R.I., and Pober, J.S. (1987). Composition of the von Willebrand factor storage organelle (Weibel-Palade body) isolated from cultured human umbilical vein endothelial cells. J. Cell Biol. *104*, 1423–1433.

Geng, J.G., Bevilacqua, M.P., Moore, K.L., McIntyre, T.M., Prescott, S.M., Kim, J.M., Bliss, G.A., Zimmerman, G.A., and McEver, R.P. (1990). Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature *343*, 757–760.

Goldstein, J.L., Brown, M.S., Anderson, R.W.G., Russel, D.W., and Schneider, W.J. (1985). Receptor mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. *1*, 1–39.

Green, S.A., and Kelly, R.B. (1992). Low density lipoprotein receptor and cation-independent mannose 6-phosphate receptor are transported from the cell surface to the Golgi apparatus at equal rates in PC12 cells. J. Cell Biol. *117*, 47–55.

Griffiths, G., Back, R., Marsh, M. (1989). A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J. Cell Biol. *109*, 2703–2720.

Hamburger, S.A., and McEver, R.P. (1990). GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood *75*, 550–554.

Harter, C., and Mellman, I. (1992). Transport of the lysosomal membrane glycoprotein 1gp120 (1gp-A) to lysosomes does not require appearance on the plasma membrane. J. Cell Biol. *117*, 311–325.

Hattori, R., Hamilton, K.K., Fugate, R.D., McEver, R.P., and Sims, P.J. (1989). Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J. Biol. Chem. *264*, 7768–7771.

Hopkins, C.R., and Trowbridge, I.S. (1983). Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J. Cell Biol. *97*, 508–521.

Johnston, G.I., Cook, R.G., and McEver, R.P. (1989). Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell *56*, 1033–1044.

Koedam, J.A., Cramer, E.M., Briend, E., Furie, B., Furie, B.C., and Wagner, D.D. (1992). P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. J. Cell Biol. *116*, 617–625.

Ktistakis, N.T., Thomas, D.'N., and Roth, M.G. (1990). Characteristics of the tyrosine recognition signal for internalization of transmembrane surface glycoproteins. J. Cell Biol. *111*, 1393–1407.

Larsen, E., Celi, A., Gilbert, G.E., Furie, B.C., Erban, J.K., Bonfanti, R., Wagner, D.D., and Furie, B. (1989). PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell *59*, 305–312.

Larsen, G.R., Sako, D., Ahern, T.J., Shaffer, M., Erban, J., Sajer, S.A., Gibson, R.M., Wagner, D.D., Furie, B.C., and Furie, B. (1992). P-selectin and E-selectin: distinct but overlapping leukocyte ligand specificities. J. Biol. Chem. *267*, 11104–11110.

Lasky, L.A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science *258*, 964–968.

Lawrence, M.B., and Springer, T.A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859–873.

Lobel, P., Fujimoto, K., Ye, R.D., Griffiths, G., and Kornfeld, S. (1989). Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. Cell *57*, 787–796.

Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I., and Helenius, A. (1987). Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. J. Cell Biol. *104*, 875–886.

Matsuda, H., and Sugiura, S. (1970). Ultrastructure of "tubular body" in the endothelial cells of the ocular blood vessel. Invest. Opthalmol. *9*, 919–925.

Matter, K., Hunziker, W., and Mellman, I. (1992). Basolateral sorting of LDL receptor in MDCK cells: the cytoplasmic domain contains two tyrosine-dependent targeting determinants. Cell *71*, 741–753.

Mayadas, T.N., and Wagner, D.D. (1989). In vitro multimerization of von Willebrand factor is triggered by low pH: importance of the propolypeptide and free sulfhydryls. J. Biol. Chem. *264*, 13497–13503.

McEver, R.P., Beckstead, J.H., Moore, K.L., Marshall-Carlson, L., and Bainton, D.F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. J. Clin. Invest. *84*, 92–99.

Moore, K.L., and Thompson, L.F. (1992). P-selectin (CD62) binds to subpopulations of human memory T lymphocytes and natural killer cells. Biochem. Biophys. Res. Commun. *186*, 173–181.

Östman, A., Thyberg, J., Westermark, B., and Heldin, C.-H. (1992). PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly. J. Cell Biol. *118*, 509–519.

Patzak, A., and Winkler, H. (1986). Exocytotic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling. J. Cell Biol. *102*, 510–515.

Picker, L.J., Kishimoto, T.K., Smith, C.W., Warnock, R.A., and Butcher, E.C. (1991). ELAM-1 is an adhesion molecule for skin-homing T cells. Nature *349*, 796–799.

Porter, R.R. (1959). The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J. *73*, 119–127.

Rice, E.G., and Bevilacqua, M.P. (1989). An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science *246*, 1303–1306.

Sellinger, O.Z., Beaufay, H., Jacques, P., Doyen, A., and deDuve, C. (1960). Tissue fractionation studies. 15. Intracellular distribution and properties of β-N-acetylglucosaminidase and β-galactosidase in rat liver. Biochem. J. *74*, 450–456.

Shimizu, Y., Shaw, S., Graber, N., Gopal, T.V., Horgan, K.J., Van Seventer, G.A., and Newman, W. (1991). Activation-independent binding of human memory T cells to adhesion molecule ELAM-1. Nature *349*, 799–802.

Sporn, L.A., Marder, V.J., and Wagner, D.D. (1986). Inducible secretion of large biologically potent von Willebrand factor multimers. Cell *46*, 185–190.

Springer, T.A. (1990). Adhesion receptors of the immune system. Nature *346*, 425–434.

Stenberg, P.E., McEver, R.P., Shuman, M.A., Jacques, Y.V., and Bainton, D.F. (1985). A platelet alpha-granule membrane protein (GMP140) is expressed on the plasma membrane after activation. J. Cell Biol. *101*, 880–886.

Stone, J.P., and Wagner, D.D. (1993). P-selectin mediates adhesion to neuroblastoma and small cell lung cancer. J. Clin. Invest. (*in press*).

Suchard, S.J., Corcoran, J.J., Pressman, B.C., and Rubin, R.W. (1981). Evidence for secretory granule membrane recycling in cultured adrenal chromaffin cells. Cell Biol. Int. Rep. *5*, 953–962.

Sudhof, T.C., and Jahn, R. (1991). Proteins of synaptic vesicles involved in exocytosis and membrane recycling. Neuron *6*, 665–677.

Tooze, S.A., Flatmark, T., Tooze, J., and Huttner, W.B. (1991). Characterization of the immature secretory granule, an intermediate in granule biogenesis. J. Cell Biol. *115*, 1491–1503.

Vaux, D. (1992). The structure of an endocytosis signal. Trends Cell Biol. *2*, 189–192.

Vischer, U.M., and Wagner, D.D. (1993). CD63 is a component of Wiebel-Palade bodies of human endothelial cells. Blood (*in press*).

von Asmuth, E.J.U., Smeets, E.F., Ginsel, L.A., Onderwater, J.J.M., Leeuwenberg, J.F.M., and Buurman, W.A. (1992). Evidence for endocytosis of E-selectin in human endothelial cells. Eur. J. Immunol. *22*, 2519–2526.

Wagner, D.D., Saffaripour, S., Bonfanti, R., Sadler, J.E., Cramer, E.M., Chapman, B., and Mayadas, T.N. (1991). Induction of specific storage organelles by von Willebrand factor propolypeptide. Cell *64*, 403–413.

Wagner, D.D., Olmsted, J.B., and Marder, V.J. (1982). Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. J. Cell Biol. *95*, 355–360.

Wagner, D.D. (1990). Cell biology of von Willebrand factor. Annu. Rev. Cell Biol. *6*, 217–246.

Weller, A., Isenmann, S., and Vestweber, D. (1992). Cloning of the mouse endothelial selectins: expression of both E- and P-selectin is inducible by tumor necrosis factor. J. Biol. Chem. *267*, 15176–15182.

Wong, S.H., Low, S.H., and Hong, W. (1992). The 17-residue transmembrane domain of β-galactoside α2,6-sialyltransferase is sufficient for Golgi retention. J. Cell Biol. *117*, 245–258.

Yokode, M., Pathak, R.K., Hammer, R.E., Brown, M.S., Goldstein, J.L., and Anderson, R.G.W. (1992). Cytoplasmic sequence required for basolateral targeting of LDL receptor in livers of transgenic mice. J. Cell Biol. *117*, 39–46.