Role of P-Selectin Cytoplasmic Domain in Granular Targeting In Vivo and in Early Inflammatory Responses

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Abstract. P-selectin is an adhesion receptor for leukocytes expressed on activated platelets and endothelial cells. The cytoplasmic domain of P-selectin was shown in vitro to contain signals required for both the sorting of this protein into storage granules and its internalization from the plasma membrane. To evaluate in vivo the role of the regulated secretion of P-selectin, we have generated a mouse that expresses P-selectin lacking the cytoplasmic domain (ΔCT mice). The deletion did not affect the sorting of P-selectin into α-granules of platelets but severely compromised the storage of P-selectin in endothelial cells. Unstored P-selectin was proteolytically shed from the plasma membrane, resulting in increased levels of soluble P-selectin in the plasma. The ΔCT–P-selectin appeared capable of mediating cell adhesion as it supported leukocyte rolling in the mutant mice. However, a secretagogue failed to upregulate leukocyte rolling in the ΔCT mice, indicating an absence of a releasable storage pool of P-selectin in the endothelium. Furthermore, the neutrophil influx into the inflamed peritoneum was only 30% of the wild-type level 2 h after stimulation. Our results suggest that different sorting mechanisms for P-selectin are used in platelets and endothelial cells and that the storage pool of P-selectin in endothelial cells is functionally important during early stages of inflammation.

Key words: P-selectin • granular targeting • platelets • endothelium • cytoplasmic domain

P-SELECTIN, together with E- and L-selectin, constitute the three members of the selectin family. All three selectin molecules contain a lectin domain at their NH_2 terminus, followed by an EGF-like domain, a variable number of complement binding-like repeats, a transmembrane domain, and a short cytoplasmic tail (Bevilacqua et al., 1989; Johnston et al., 1989; Tedder et al., 1989). Cloning of the selectin genes has revealed structural and functional conservation between the respective human and murine selectins (Becker-Andre et al., 1992; Weller et al., 1992). Numerous studies in recent years have shown that the major function of selectins is to mediate the binding of leukocytes to activated endothelium or platelets. Selectin-deficient mice generated through gene targeting exhibit various defects in inflammation, lymphocyte homing, and hematopoiesis (for review see Springer, 1995; Frenette and Wagner, 1997).

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Despite the structural similarity, each of the selectins has a unique pattern of expression. E-selectin is expressed on activated endothelial cells through *de novo* synthesis upon stimulation (Bevilacqua et al., 1987). L-selectin, on the other hand, is constitutively expressed on leukocytes and is shed from the cell membrane after activation of these cells (Tedder et al., 1989). Unlike E- or L-selectin, P-selectin is constitutively present in α-granules of platelets (Stenberg et al., 1985; Berman et al., 1986), and Weibel-Palade bodies of endothelial cells, and is only translocated to the cell surface after activation (Bonfanti et al., 1989; McEver et al., 1989). The cell surface expression of P-selectin is tightly regulated in both platelets and endothelial cells. Studies have shown that P-selectin is rapidly shed from activated platelets in vivo (Michelson et al., 1996; Berger et al., 1998). In endothelial cells, the kinetics of P-selectin expression varies depending on the secretagogue or agonists used. During acute inflammation, transient surface expression of P-selectin can be induced by histamine, thrombin, or complement components (Hattori et al., 1989; Geng et al., 1990; Subramaniam et al., 1993; Foreman et al., 1994), and the expressed P-selectin is then rapidly internalized and resorted to Weibel-Palade bodies

or lysosomes (Hattori et al., 1989; Subramaniam et al., 1993; Green et al., 1994). Prolonged surface expression of P-selectin on endothelial cells has been observed when these cells are stimulated with oxygen radicals or cytokines such as interleukin-3 (Patel et al., 1991; Khew-Goodall et al., 1996).

The cytoplasmic domain (CT)[1] of P-selectin contains sequence elements required for both sorting of the protein into storage granules and its internalization from the plasma membrane. Deletion of this domain leads to surface expression of P-selectin in AtT-20 cells after transfection whereas the transfected wild-type P-selectin is sorted to storage granules in these cells (Disdier et al., 1992; Koedam et al., 1992). Tissue factor, normally a secreted protein, is redirected to storage granules when its cytoplasmic domain is replaced with that of P-selectin (Disdier et al., 1992). Rather than a single element, several stretches of amino acids in the CT appear to be involved in the sorting process (Disdier et al., 1992; Norcott et al., 1996), and the transmembrane domain of P-selectin further improves the efficiency of granular targeting (Fleming et al., 1998). The sequences required for P-selectin internalization also appear to be distributed throughout its CT. Various mutations and deletions of amino acids within the CT can lead to decreased efficiency of internalization (Setiadi et al., 1995). However, in transfected neuroendocrine cells, significant surface accumulation of P-selectin occurred only when the majority of the CT was deleted (Norcott et al., 1996).

The purpose of this study was to evaluate the role of the CT in P-selectin function as well as the importance of the regulated secretion of P-selectin in vivo. We have generated a mouse that expresses P-selectin without the CT by gene replacement through homologous recombination in embryonic stem (ES) cells. We have observed that different sorting mechanisms for P-selectin may be used in platelets and endothelial cells and that P-selectin with a deleted cytoplasmic domain appears capable of mediating the binding of leukocytes to platelets in vitro and to endothelial cells in vivo. In addition, elevated levels of soluble P-selectin were detected in the plasma of the mutant mice which presumably resulted from cleavage of the constitutively expressed endothelial P-selectin.

Materials and Methods

Construction of the Targeting Vector

A mouse genomic library made from the livers of Black Agouti 129Sv strain (gift of R. Jaenisch, Massachusetts Institute of Technology, Cambridge, MA) was screened with a mouse cDNA probe spanning CR8, transmembrane domain, and C1 and C2 exons obtained from D. Vestweber (Münster, Germany). The genomic clone containing the 3′ end of the P-selectin gene was subcloned into Bluescript KS vector (Stratagene, La Jolla, CA). Two stop codons and XhoI-EcoRI-XbaI restriction sites were inserted into the genomic clone by PCR amplification using primers complementary to a sequence within the intron after CR8 and the 3′ end of exon TM (see Fig. 1 *a*) which encoded the transmembrane domain and the first seven amino acids of the CT. The 3′ end of the human growth hormone (hGH) gene was then inserted into the EcoRI site introduced by PCR. A 1.7-kb neomycin resistance gene with the phosphoglycerate kinase promoter (PGK*neo*) was inserted immediately after the hGH gene fragment. The resulting fragment contained 5 and 2.5 kb of P-selectin genomic sequences upstream and downstream of the insertions, respectively, and was cloned into a Bluescript vector containing a herpes simplex virus-thymidine kinase (HSV-TK) cassette (see Fig. 1 *a*). The final construct was linearized with NotI for transfection.

ES Cell Transfection, Selection, and Genotyping

D3 ES cells derived from Black Agouti 129Sv mouse embryos (Doetschman et al., 1985) were cultured on mitotically inactivated fibroblasts in standard ES cell media (George and Hynes, 1994), and electroporated with the linearized DNA construct. Transfected ES cells were selected for occurrence of homologous recombination by culturing in the presence of G418 and gancyclovir (Mayadas et al., 1993). Genomic DNA isolated from ES cell clones that survived the selection were digested with EcoRI and separated on 1% agarose gels. A standard Southern blot protocol was used as described previously (Mayadas et al., 1993), and targeted ES cell clones were identified by using a probe of a 350-bp genomic DNA fragment downstream of the targeting construct (see Fig. 1 *a*). DNA from positive clones was also probed with a fragment of the neomycin resistance gene to ensure a single integration of the targeting construct.

Generation and Breeding of Chimeric Mice

ES cells with one mutated allele of the P-selectin gene were microinjected into the blastocoel of 3.5-d-old blastocysts isolated from C57BL/6J mice. The injected blastocysts were then implanted into pseudopregnant females (Bradley, 1987). Chimeric males obtained were bred with C57BL/6J females and agouti progeny were genotyped by Southern blot analysis of tail biopsies.

Northern Blot Analysis

Total RNA from lung tissue was harvested from mice treated with LPS (i.p., 20 μg/g body weight) for 3.5 h using RNA-stat 60 (Tel-test B Inc., Friendswood, TX). 20–25 μg of total RNA was electrophoresed on a 1.2% agarose gel containing 0.66 M of formaldehyde and then transferred to a nylon membrane (Schleicher & Shuell, Keene, NH). P-selectin transcripts were detected by probing with a 1.6-kb cDNA fragment encoding the 3′ half of murine P-selectin (gift of D. Vestweber). Antisense oligonucleotides corresponding to almost the entire P-selectin transmembrane domain (66 bp) or cytoplasmic domain which included sequences from both exons C1 and C2 (72 bp), or most of the hGH 3′-UTR sequence (63 bp) were also used as probes to further characterize mRNA species.

Western Blot Analysis

Blood samples were collected by retro-orbital plexus bleeding into polypropylene tubes containing a 0.1× final volume of ACD (38 mM citric acid, 75 mM trisodium citrate, and 100 mM dextrose). Platelet-rich plasma (PRP) were prepared by centrifugation of the blood samples at 100 *g* for 5 min and gently collecting the supernatant without disturbing the buffy coat. Platelets were obtained from PRP by centrifugation at 900 *g* for 5 min and lysed in an SDS sample buffer (65 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS). Platelet lysates or plasma samples were boiled for 5 min, separated on a 7.5% SDS-polyacrylamide gel, and then transferred to PVDF membranes (Millipore, Bedford, MA). Blots were then probed with polyclonal antibodies against human P-selectin which also recognize mouse P-selectin (PharMingen, San Diego, CA) or a peptide sequence in the P-selectin cytoplasmic domain (gift of M. Berndt, Clayton, Victoria, Australia). The antibody bound to P-selectin was detected with a horseradish peroxidase-conjugated goat anti–rabbit IgG (Bio-Rad, Hercules, CA) and an enhanced chemiluminescence kit (Sigma Chemical Co., St. Louis, MO).

Immunofluorescence Staining

Blood smears or frozen tissue sections (8 μm) were fixed in 3.7% (vol/vol) formaldehyde and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS) without divalent cations. Samples were then incubated for 30 min with a rabbit anti-human P-selectin antibody diluted at 1:50 (gift of M. Berndt), followed by a 30-min incubation with a FITC-labeled

1. *Abbreviations used in this paper:* ΔCT, P-selectin with a deleted cytoplasmic domain; CT, cytoplasmic domain; ES, embryonic stem; hGH, human growth hormone; LPS, lipopolysaccharide; PBS-T, PBS containing 0.1% Tween 20; PRP, platelet-rich plasma; vWF, von Willebrand factor.

goat anti–rabbit antibody at 1:500 dilution (Cappel, Durham, NC). P-selectin–null platelets or sections were used as negative controls. For double staining of P-selectin and von Willebrand factor (vWF), samples were first stained with a sheep anti–human vWF antibody at 1:50 dilution (Biodesign International, Kennebunkport, ME) followed by an FITC-conjugated donkey anti–sheep IgG at 1:200 dilution (Jackson ImmunoResearch Labs, West Grove, PA). The samples were then stained for P-selectin as stated above except that a rhodamine-conjugated donkey anti–rabbit IgG was used as the secondary antibody (1:200) (Jackson ImmunoResearch Labs).

Flow Cytometry

Resting platelets were collected from PRP containing PGE1 by centrifugation at 900 *g* for 5 min. Platelets were resuspended in Pipes buffer (25 mM Pipes, 137 mM NaCl, 4 mM KCl, 0.1% dextrose, pH 7.4) and incubated with a rabbit anti–P-selectin antibody (provided by M. Berndt) at 1:100 dilution and FITC-labeled goat anti–rabbit antibody (Cappel) at 1:500 dilution. For activation, platelets were washed three times with Pipes buffer, pH 7.4, and incubated with 0.5 U/ml of thrombin for 15 min at 37°C, followed by labeling with primary and secondary antibodies. 10,000 platelets were analyzed for each sample.

Rosetting Assay

The adhesion of platelets to HL-60 cells (American Type Culture Collection, Rockville, MD) was performed as described previously (Larsen et al., 1989), except that 40-μl aliquots of platelets and cell suspensions were used in each assay. An HL-60 cell bound to two or more platelets was counted as one adhesion event. In some experiments, rosetting was done in the presence of 5 mM EDTA. In other experiments, HL-60 cells were treated with neuraminidase (0.1 U/ml) at 37°C for 60 min, and then washed three times before incubation with platelets.

Electron Microscopy

Platelets in whole blood were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at 22°C and then isolated from PRP. The platelets were washed three times with 0.1 M phosphate buffer and then embedded in sucrose and frozen in liquid nitrogen (Slot et al., 1988). Sucrose-embedded samples were cut at −100°C using a Reichert ultramicrotome Ultracut E and a FC4E cold chamber (Leica, Deerfield, IL). The immunochemical reactions were then performed on ultrathin sections collected on grids (Slot et al., 1988). In brief, the sections were labeled by incubation with a polyclonal anti–P-selectin antibody diluted in PBS containing 1% of bovine serum albumin (Sigma Chemical Co.) for 20 min at 22°C, washed, and then incubated with gold-conjugated (10 nm) goat anti–rabbit IgG (Amersham, Buckinghamshire, UK) for 20 min at room temperature. The sections were counterstained with 2% uranyl acetate, pH 7.0, and methyl cellulose uranyl. Samples were observed on a JEOL 1200EX electron microscope (JEOL USA, Peabody, MA).

Primary Mouse Lung and Brain Endothelial Cell Culture

For each lung endothelial cell preparation, lung tissues were collected from three or more wild-type or mutant mice, washed in DME, and then minced into 1–2-mm pieces. The puree was then digested with 20 ml of 0.1% collagenase A (Sigma Chemical Co.) at 37°C for 1 h. The cellular digest was filtered through a 40-μm nylon mesh, centrifuged at 100 *g* for 10 min, and then the cells were plated in F12 (HAM) medium (GIBCO BRL, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS), 0.2 U/ml heparin, and 5 μg/ml endothelial mitogens (Biomedical Technologies, Stoughton, MA). 48 h later, the plates were washed with PBS and fresh culture medium was added. Dynabeads coated with sheep anti–rat IgG (Dynal, Lake Success, NY) were incubated with a rat anti–mouse intercellular adhesion molecule-2 IgG (PharMingen) at 4°C overnight, washed three times with 2% FBS in PBS, and then added to the plated cells. After 1 h of incubation at 37°C, the plates were washed with PBS and trypsinized to collect the cells. Cells bound to the coated beads were recovered through a magnetic field, washed, and then plated on coverslips coated with 1% gelatin (Sigma Chemical Co.). Brain microvascular endothelial cells were isolated from 10 wild-type or mutant mice as described previously (Barkalow et al., 1996*b*). In brief, cerebral cortex homogenates were subject to two successive collagenase digestions followed by Percoll gradient density centrifugation. Capillary fragments were collected, washed, and then plated onto fibronectin-coated culture dishes or Lab-Tec chamber slides.

Analysis of Leukocyte Rolling by Intravital Microscopy

Mice were anesthetized and mesentery was prepared as described (Johnson et al., 1995). In venules 25–35 μm in size, baseline leukocyte rolling was recorded in the first 10 min after exteriorization. Subsequently, 30 μl of 10 μM solution of A23187, a calcium ionophore, was applied to the exposed mesentery, and leukocyte–endothelium interactions were recorded for another 10 min. Recorded images were analyzed as follows: the number of cells passing a given plane perpendicular to the vessel axis in 1 min was defined as a 1-min count. Baseline rolling and rolling after activation for each mouse were determined by taking the average of four 1-min counts during the first and the second 10-min recording, respectively.

Thioglycollate-induced Peritonitis

As described previously (Mayadas et al., 1993), each mouse was injected intraperitoneally with 1 ml of 3% thioglycollate (Sigma Chemical Co.). Peritoneal lavage was harvested in 9 ml of PBS containing 0.1% BSA, 0.5 mM EDTA, and 10 U/ml heparin. Total cells in the lavage were counted by a Coulter counter. Cytospin preparations of the lavage were stained with Wright's stain and differentially counted to determine the percentage of neutrophils.

ELISA for Soluble P-Selectin

Blood samples from retro-orbital plexus bleeding were collected in polypropylene tubes containing 0.1 volume of ACD, and centrifuged immediately at 1,000 *g* for 15 min. Plasma was collected and centrifuged at 16,000 *g* for 10 min to remove cell debris. Microtiter plates were coated overnight at 4°C with a monoclonal anti-mouse P-selectin antibody (RB 40.34; PharMingen) at 2 μg/ml in PBS. The plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and 0.5% BSA and blocked with PBS-T containing 1% BSA for 30 min at room temperature. Plasma samples were diluted in PBS-T containing 0.5% BSA and 0.5% gelatin, and were incubated in coated wells for 2 h at 37°C. After washing, a biotinylated rabbit anti–P-selectin antibody (PharMingen) was added to the plates and incubated for 2 h. ExtrAvidin-conjugated alkaline phosphatase was added after three washes and the activity was revealed with its substrate *p*-nitrophenyl phosphate (Sigma Chemical Co.). The plates were read at 405 nm in an Epson LX-300 ELISA reader (Dynatech Laboratories, Chantilly, VA).

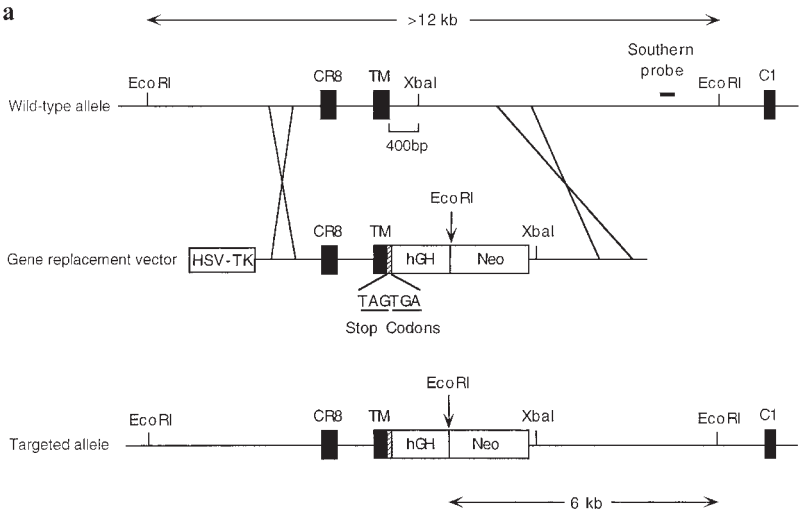
Statistical Analysis

Statistical significance was assessed by the Student's *t* test and data were presented as mean ± SEM.

Results

Generation of Mice Expressing P-Selectin with a Deleted Cytoplasmic Domain

A P-selectin genomic clone containing exons 13 and 14 was isolated from a mouse genomic library. Exon 13 encodes the transmembrane domain and the first seven amino acids of the CT, and exon 14 encodes the next 10 amino acids of the CT. To obtain P-selectin without the cytoplasmic tail, two translation stop codons were inserted after the first three amino acids of the CT. After the stop codons, a 620-bp fragment from the 3′ hGH gene was inserted to provide transcription termination and polyadenylation signals (Neufeld et al., 1988). The gene replacement vector (Fig. 1 *a*) also contained a PGK*neo* sequence and a HSV-TK cassette. After electroporation of D3 ES cells, clones resistant to G418 and gancyclovir were screened for targeted alleles by Southern blot analysis. Chimeric mice derived from ES cells containing the mu-



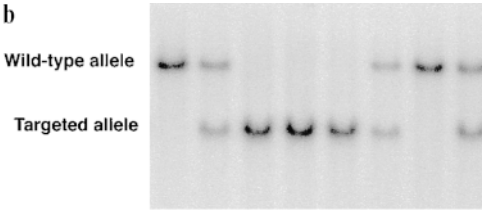


Figure 1. Gene replacement strategy and Southern blot analysis of progeny from heterozygous crosses. (*a*) The wild-type P-selectin allele, the replacement vector, and the mutated allele are shown. Exon TM encodes the transmembrane domain and the first seven amino acids of the CT of P-selectin. Using PCR amplification, two stop codons and XhoI-EcoRI-XbaI restriction sites were inserted after the first three amino acids of the CT. A 620-bp fragment containing 3′-UTR of the hGH gene and the PGK*neo* cassette were inserted into the EcoRI site introduced by PCR. A 320-bp fragment immediately following the TM exon and before the XbaI site was deleted in the targeting construct. A probe flanking the 3′-end of the vector was used for Southern blot analysis of ES cell clones and tail biopsies of the progeny. The probe detects a EcoRI fragment of >12 kb from the wild-type allele and a 6-kb fragment from the targeted allele since an EcoRI site is introduced between the hGH and PGK*neo* gene. (*b*) Representative Southern blot analysis of tail biopsies of progeny from heterozygous crosses. Genomic DNA was digested with EcoRI, electrophoresed, and then blotted. The fragments from wild-type and mutant alleles are as indicated.

tated P-selectin gene were bred to C57BL/6J females to achieve germ-line transmission. Mice homozygous for the mutation (ΔCT mice) (Fig. 1 *b*) appeared grossly normal with normal blood counts including total leukocytes, platelets, and neutrophils (data not shown).

Verification of the Expression of the Mutant Gene

To determine whether the mutated P-selectin gene was expressed in homozygous mutant mice, total RNA was extracted from the lungs of wild-type, heterozygous, and homozygous animals after treatment with lipopolysaccharide (LPS) for 3.5 h to stimulate P-selectin synthesis (Sanders et al., 1992). A Northern blot probed with murine P-selectin cDNA revealed a 3-kb transcript in wild-type mice as expected (Fig. 2). A truncated transcript of 2.2 kb encoding domains from the NH_2-terminal to the transmembrane domain with the addition of the hGH 3′-UTR was predicted to be expressed by heterozygous as well as the homozygous mice. As shown in Fig. 2, indeed both the wild-type and the truncated transcripts were present in heterozygous mice. In homozygous mice, the truncated mRNA species represented the majority of the message detected by the P-selectin cDNA probe. However, the P-selectin cDNA probe also detected two minor bands at higher molecular weight (Fig. 2, *asterisk*) in homozygous mutants. Subsequent probing with oligo antisense DNA probes revealed that these upper bands contained sequences of the transmembrane and cytoplasmic domain, and the 3′-UTR of the hGH gene. These results show that some read-through transcription beyond the hGH transcription terminator must occur at a reduced level to include sequences from the neomycin cassette and C1 and C2 exon. These transcripts could then be spliced to produce mRNAs which include sequences of C1 and C2 exons. Although the exact splicing events cannot readily be determined, one can deduce the following possible outcomes. First, any mRNA that correctly splices exon CR8 and exon TM will necessarily terminate translation at the termination codons introduced during the mutagenesis, whatever other sequences follow in the mRNA. All such mRNAs will therefore encode the truncated membrane-bound form without CT as anticipated. Any aberrant splicing events that exclude the TM will yield secreted proteins. The most readily conceived would be an aberrant splice from CR8 to C1. If such a splice were to occur, the reading frame would be preserved so that any resulting protein would react with the antibody against the CT. The same would be the case if any other CR exon were to splice to C1 since all CR exons are in the same phase (Johnston et al., 1990; Sanders et al., 1992). As will be discussed below, no protein containing the P-selectin CT was found in the ΔCT mice.

The P-selectin protein produced from the mutated gene was first analyzed by Western blot analysis of platelet lysates. Using a polyclonal anti–P-selectin antibody, a band at ~140 kD was detected in both wild-type and ΔCT platelet lysates whereas it was absent in P-selectin–null platelets (Fig. 3 *a*). A parallel probing using the polyclonal antibody against a peptide of the P-selectin CT revealed no signal at the molecular mass of P-selectin in ΔCT platelets whereas it was present in the wild-type (Fig. 3 *b*). Similar Western blot analysis of lung tissue lysates indicated that the endothelium-derived P-selectin in the mutant mice did not contain the CT either (data not shown). It is noteworthy that the mutant P-selectin detected in platelets and lung tissues was only slightly smaller than full-length

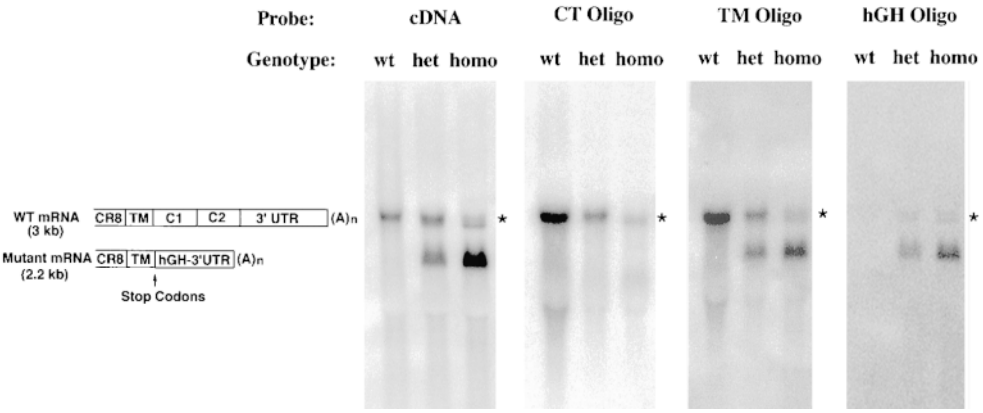


Figure 2. Northern blot analysis of RNA samples (25 μg/lane) from lung tissues of mice treated with LPS for 3.5 h. Wild-type (*wt*), heterozygous (*het*), and homozygous (*homo*) mutants are as indicated. The cDNA probe is a 1.6-kb fragment from the 3′ half of P-selectin. TM and CT probes are 66-base and 72-base antisense oligonucleotides of the respective domains. The hGH probe is a 63-base oligonucleotide from hGH 3′-UTR. The diagram depicts the predicted wild-type and mutant mRNA. *Asterisk*, the aberrant minor mRNA species discussed in the text.

P-selectin, entirely consistent with its being the ΔCT form predicted.

Localization of ΔCT–P-Selectin in Platelets

The level of P-selectin expressed on the surfaces of platelets was determined by FACS® analysis. Fig. 4 illustrates typical fluorescence histograms of P-selectin staining of platelets. Very little P-selectin was detected on the surface of ΔCT platelets in the resting state, as was the case for wild-type platelets (Fig. 4). After activation by thrombin, ~90% of the platelets, from both the mutant and the wild-type animals, expressed P-selectin on their surfaces (Fig. 4). In addition, similar means of fluorescence intensities were observed in platelets of the two genotypes. These results indicate that, similar to wild-type platelets, the resting ΔCT platelets do not express significant amounts of P-selectin on the plasma membrane, and that levels of P-selectin stored in a releasable pool inside the platelets are comparable in the wild-type and ΔCT platelets.

Immunofluorescence staining of blood smears revealed a granular staining pattern for P-selectin in ΔCT platelets which was similar to that of wild-type platelets (data not shown). Furthermore, immunolocalization by electron microscopy was performed to locate precisely the ΔCT–P-selectin molecules inside the platelets. Immunogold labeling of P-selectin displayed essentially a similar distribution of gold particles in ΔCT and wild-type platelets.

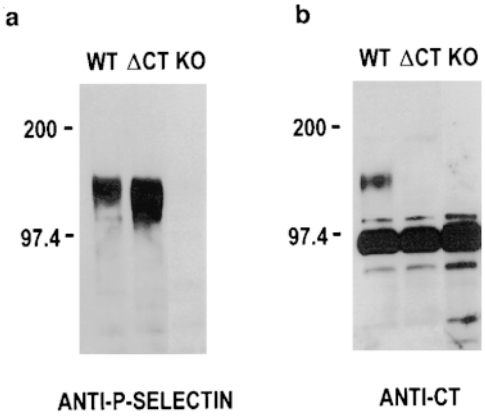


Figure 3. Western blot analysis of P-selectin expressed in platelets. Platelets from wild-type (*WT*), ΔCT, and P-selectin–knockout (*KO*) mice were lysed in SDS lysis buffer at 5×10^9/ml. 10 μl of lysates were loaded in each lane of 7.5% SDS polyacrylamide gels. (*a*) The blot was probed with polyclonal antibodies against P-selectin. (*b*) A polyclonal antibody against a KLH-coupled P-selectin cytoplasmic domain peptide sequence was used for probing.

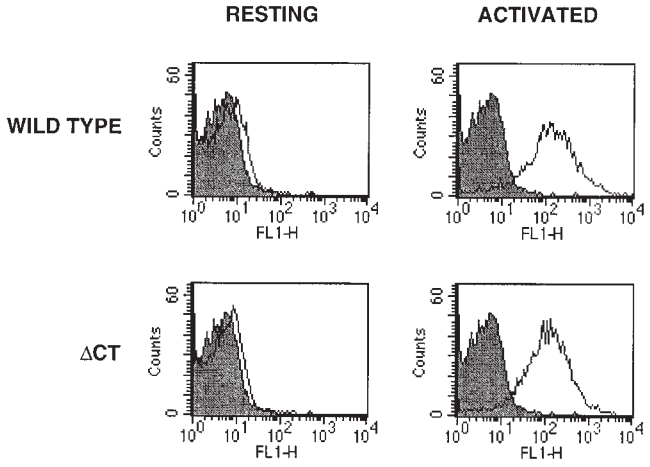


Figure 4. Flow cytometry analysis of P-selectin expression on platelets. Wild-type and ΔCT platelets were stained for membrane P-selectin and analyzed by flow cytometry. In the resting state, platelets of both genotypes displayed virtually no P-selectin on their plasma membranes. Thrombin activation induced in wild-type as well as in mutant platelets a similar increase in mean fluorescence with ~90% of P-selectin–positive platelets. Representative histograms are shown. *Shaded area*, negative control staining with only the FITC-conjugated goat anti–rabbit IgG.

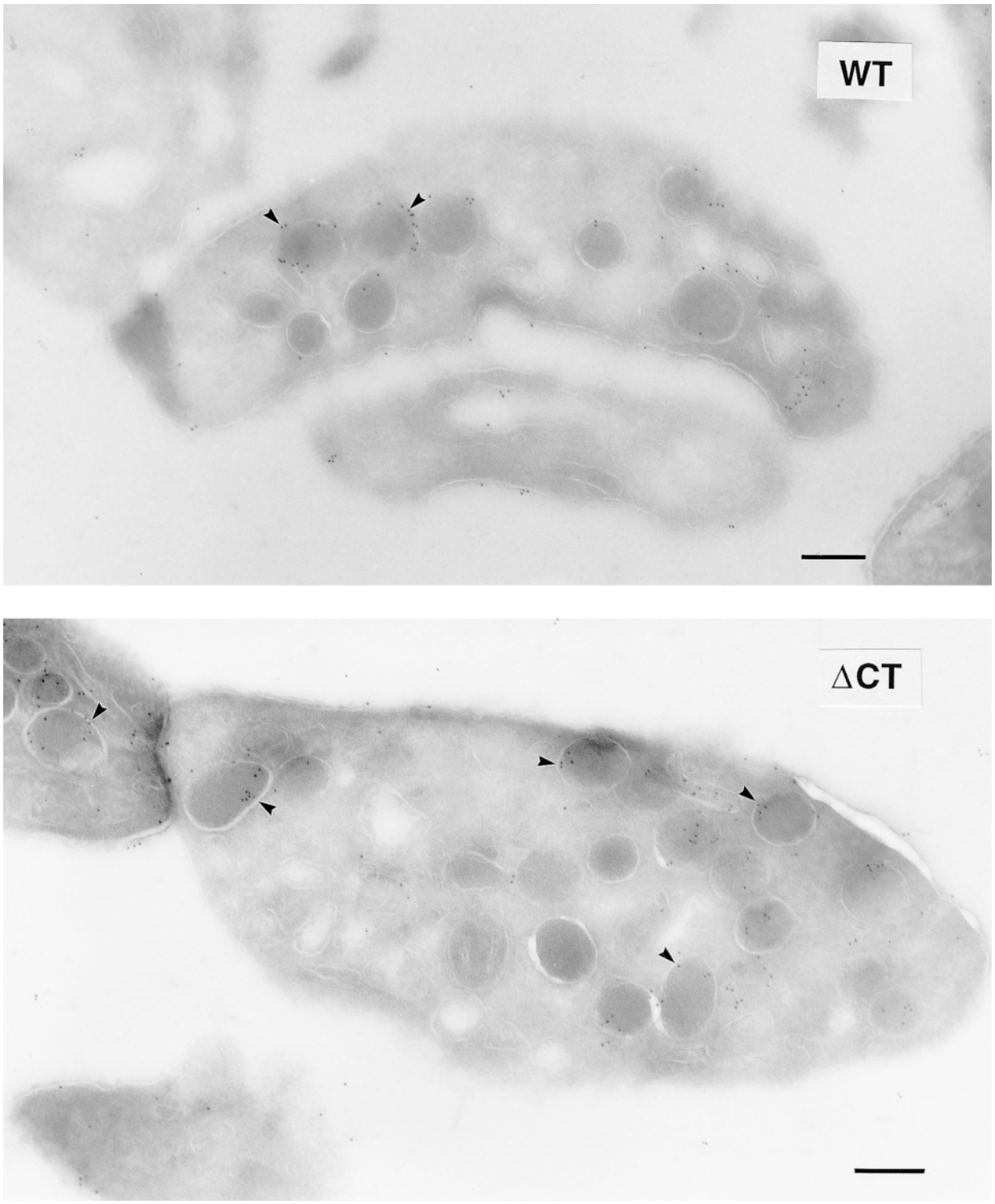


Figure 5. Localization of ΔCT–P-selectin in platelets by electron microscopy. Indirect immunogold labeling of P-selectin was performed in resting platelets from wild-type (*WT*) and ΔCT mice. Ultrathin frozen sections were stained with a rabbit antibody against P-selectin and visualized with a goat anti–rabbit IgG conjugated to 10-nm colloidal gold particles. The majority of the gold particles are associated with α-granules (*arrowheads*) in both wild-type and ΔCT platelets. A small amount of labeling was seen on the plasma membrane in both genotypes. Bars, 200 nm.

P-selectin molecules without CT were preferentially associated with the α-granules as was the case for wild-type molecules (Fig. 5). This indicates that the cytoplasmic domain is not necessary for P-selectin localization to the α-granules.

ΔCT Platelet Binding to HL-60 Cells

Previous studies have shown that the binding of activated platelets to neutrophils and monocytes is mediated by

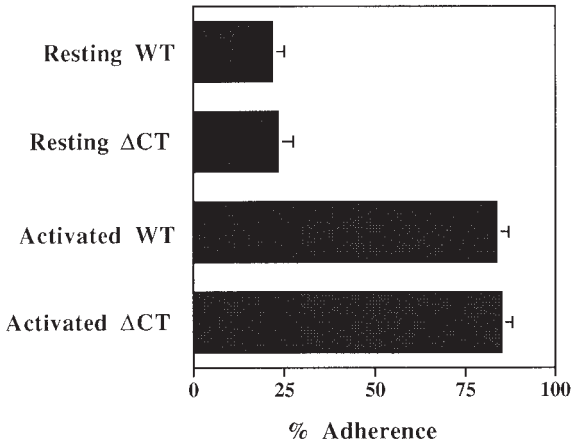


Figure 6. Interaction of resting and activated platelets with HL-60 cells. The interaction between platelets and HL-60 cells was determined under phase microscopy and the percentages of HL-60 cells bound to two or more platelets are shown. The data represent the mean of three separate experiments.

P-selectin (Larsen et al., 1989; Hamburger and McEver, 1990). The ability of ΔCT–P-selectin to mediate platelet–leukocyte interactions was assessed by a rosetting assay (Larsen et al., 1989). Resting or thrombin-activated platelets were incubated with HL-60 cells which express functional PSGL-1, the major ligand for P-selectin. The interaction between the two cell types was evaluated by light microscopy. As shown in Fig. 6, few rosetting events were observed between HL-60 cells and resting platelets of either genotype. When activated with thrombin, the ΔCT platelets bound to HL-60 cells to a similar extent as wild-type platelets. Rosetting was eliminated by the presence of EDTA or pretreatment of HL-60 cells with neuraminidase (data not shown).

Endothelial Expression of ΔCT–P-Selectin

The expression of ΔCT–P-selectin by endothelial cells in vivo was evaluated by fluorescence staining of P-selectin on frozen sections of the lung and heart. Compared with that of the wild type, the positive staining for P-selectin appeared less intense in the ΔCT vessels (data not shown). However, the limited resolution of the method does not permit differentiation of P-selectin localized in the storage granules or on the cell surface. To determine whether the P-selectin molecules without the CT were stored in Weibel-Palade bodies, lung microvascular endothelial cells were isolated and cultured in vitro. The cultured endothelial cells were double stained for P-selectin and vWF, a major component of Weibel-Palade bodies (Wagner et al.,

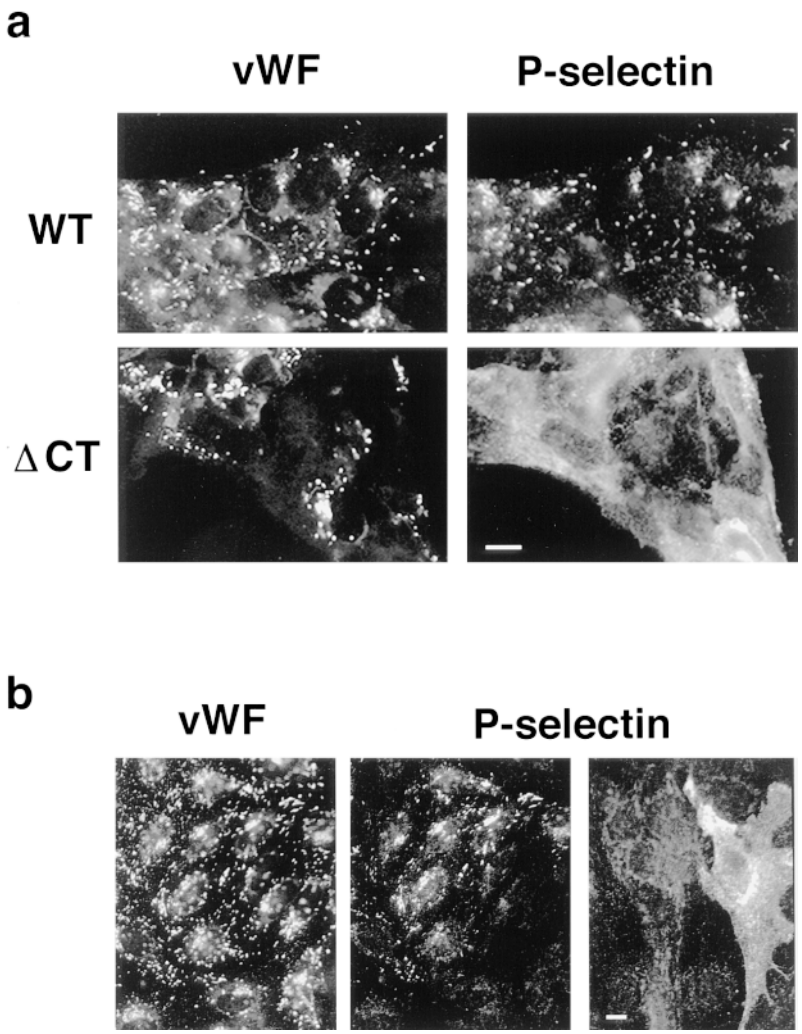


Figure 7. Immunofluorescence localization of P-selectin in cultured endothelial cells. (*a*) Cultured lung microvascular endothelial cells were double stained for P-selectin and vWF. Identical fields are shown for P-selectin and vWF staining in each genotype. In wild-type endothelial cells, P-selectin colocalized with vWF in a granular pattern typical of Weibel-Palade bodies. In ΔCT endothelial cells, strong membrane staining for P-selectin was observed. vWF staining showed normal granular localization in the mutant cells. (*b*) Cultured brain microvascular endothelial cells from ΔCT mice. *Left*, vWF localization in Weibel-Palade bodies. *Middle*, the identical field stained for P-selectin. Colocalization of P-selectin with vWF in Weibel-Palade bodies was observed. *Right*, a ΔCT brain endothelial cell with strong membrane staining for P-selectin. Bars, 3 μm.

1982). In wild-type endothelial cells, the distribution of P-selectin was colocalized with that of vWF to the Weibel-Palade bodies (Fig. 7 *a*). Meanwhile, the granular staining for P-selectin was much reduced or absent in ΔCT endothelial cells. Instead, strong membrane staining of P-selectin was observed in these cells, with vWF remaining in the Weibel-Palade bodies (Fig. 7 *a*). Similar predominant membrane staining of ΔCT–P-selectin was observed in four separate preparations of lung endothelial cell cultures. Therefore, it appears that, unlike the situation in platelets, the cytoplasmic domain plays a critical role in sorting of P-selectin into Weibel-Palade bodies in lung endothelial cells. To our surprise, some brain microvascular endothelial cells isolated from the mutant mice were capable of sorting ΔCT–P-selectin into granules. Although surface staining of ΔCT–P-selectin was still frequently observed in the brain microvascular endothelial cells (Fig. 7 *b*, *right*), the ΔCT–P-selectin was also localized in Weibel-Palade bodies in numerous cultured brain endothelial cells (Fig. 7 *b*, *middle*).

Leukocyte Rolling in the ΔCT Mice

The ability of endothelial ΔCT–P-selectin to mediate leukocyte rolling was assessed by intravital microscopy. Upon exteriorization of the mesentery, the baseline leukocyte rolling, a process shown previously to be dependent on endothelial P-selectin (Mayadas et al., 1993), was still observed in the ΔCT mice, and its frequency was comparable to that of the wild type (Fig. 8). This observation suggests that ΔCT–P-selectin expressed by endothelial cells is capable of mediating functional binding to P-selectin ligands on leukocytes in vivo. Subsequently, the venules were superfused with the calcium ionophore A23187 to induce the degranulation of Weibel-Palade bodies (Sporn et al., 1986; Mayadas et al., 1993). In wild-type mice, a twofold increase in number of rolling leukocytes was observed within 2–3 min of stimulation. In contrast, this increase did not occur in the mutant mice, suggesting the absence of a significant releasable storage pool of P-selectin in the mesenteric endothelial cells of the ΔCT mice (Fig. 8).

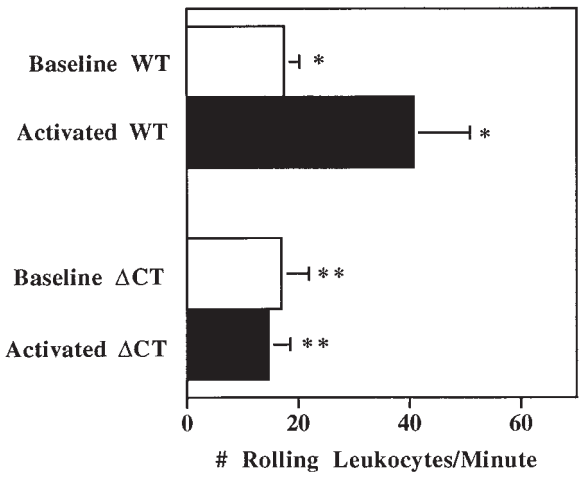


Figure 8. Analysis of leukocyte rolling in mesenteric venules. Baseline rolling was recorded during the first 10 min after exteriorization of the mesentery. The number of rolling leukocytes was quantified by counting the cells passing through a perpendicular plane in 1 min. To examine leukocyte rolling on activated endothelium, A23187 was added to the exposed mesentery and leukocyte rolling was recorded for an additional 10 min. The data represent the mean of seven mice of each genotype at 4 wk of age. *, $P < 0.05$; **, $P = 0.71$. A previous study (Mayadas, 1993) has shown that the number of rolling leukocytes per minute in P-selectin–deficient mice was minimal: <0.05 and 0.10 ± 0.06 for resting and activated venules, respectively.

Delayed Neutrophil Recruitment in ΔCT Mice in Thioglycollate-induced Peritonitis

P-selectin has been shown to play an important role in neutrophil influx during early stages of thioglycollate-induced peritonitis (Mayadas et al., 1993). The effect of the absence of P-selectin storage pool in the mesentery of the ΔCT mice was evaluated in this chemically induced inflammatory model. Thioglycollate was injected into the peritoneal cavities of mutant and wild-type mice and peritoneal lavages were collected after various time intervals. At the 2-h time point, the neutrophil influx in the ΔCT mice was only 30% of that of wild-type mice (Fig. 9). However, this defect in neutrophil emigration in the ΔCT mice was corrected by 4 h after thioglycollate injection. The number of neutrophils recruited to the peritoneal cavity remained comparable between the two genotypes at 24 and 48 h after the induction of peritonitis (Fig. 9). These results indicate that the absence of a stored pool of P-selectin affects only the early stages of inflammation.

Shedding of the Tailless P-Selectin into the Plasma

The fact that the baseline leukocyte rolling was not unusually high in ΔCT mice, combined with the weak fluorescence staining of the ΔCT vessels, suggests that the ΔCT–P-selectin delivered to the surface of endothelial cells

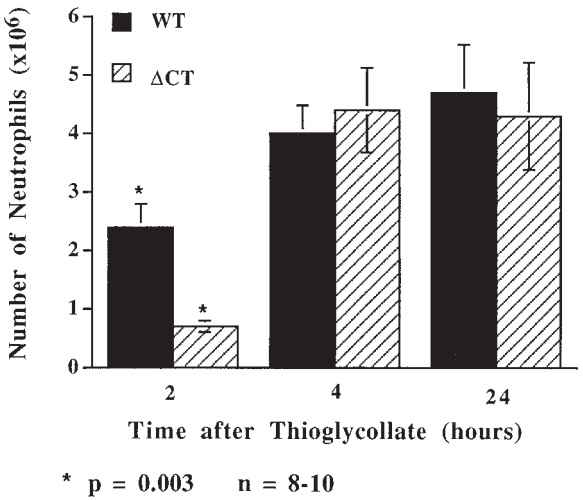


Figure 9. Neutrophil recruitment in thioglycollate-induced peritonitis. Peritoneal lavages were collected at indicated time intervals after thioglycollate injection. Total cells in the lavages were determined using a Coulter counter. Neutrophil numbers were differentially counted on Wright-Giemsa–stained cytospin preparations. Eight to ten mice of each genotype, 8–10 wk of age, were used for each time point.

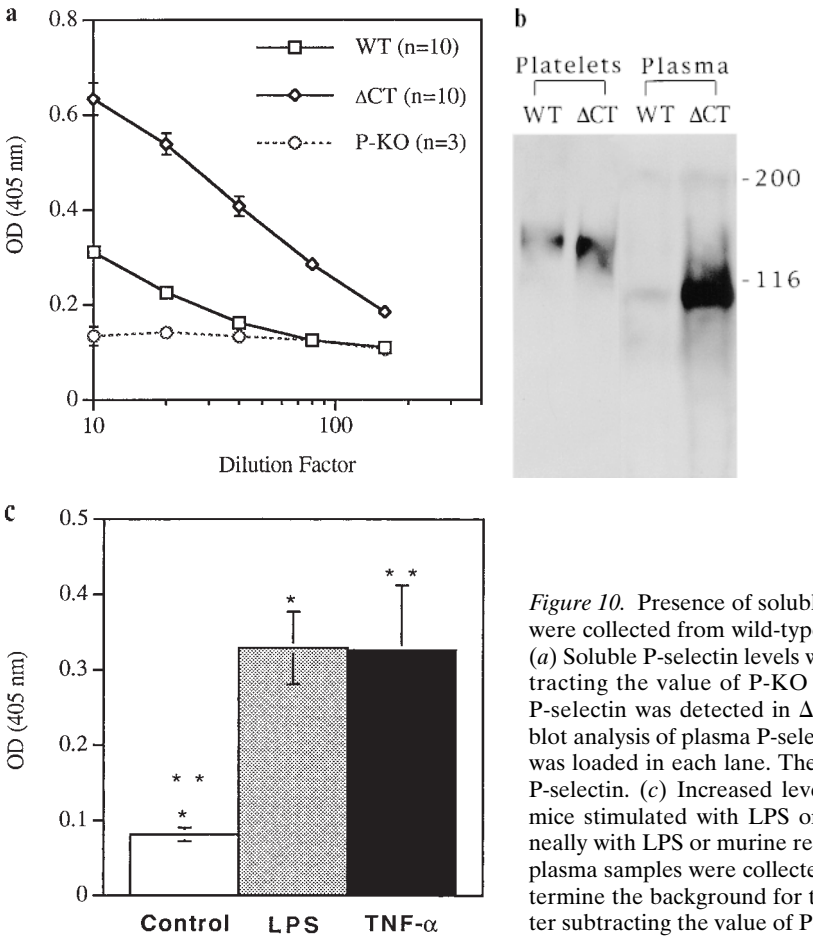


Figure 10. Presence of soluble P-selectin fragment in the plasma. Plasma samples were collected from wild-type (*WT*), ΔCT, and P-selectin–deficient (*P-KO*) mice. (*a*) Soluble P-selectin levels were determined using a sandwich ELISA. After subtracting the value of P-KO plasma, an average of fivefold increase in plasma P-selectin was detected in ΔCT mice compared with the wild-type. (*b*) Western blot analysis of plasma P-selectin. 10 μl of plasma or lysates from 5×10^7 platelets was loaded in each lane. The blot was probed with a polyclonal antibody against P-selectin. (*c*) Increased levels of soluble P-selectin in the plasma of wild-type mice stimulated with LPS or TNF-α. Wild-type mice were injected intraperitoneally with LPS or murine recombinant TNF-α (both at 20 μg/g body weight) and plasma samples were collected 4 h later. Plasma from P-KO mice was used to determine the background for the ELISA. The OD values shown were obtained after subtracting the value of P-KO plasma. *, $P < 0.002$; **, $P = 0.02$; $n = 3$ or 4.

might be removed from the plasma membrane in vivo. Indeed, using a sandwich ELISA, we have detected an over fivefold increase in the level of soluble P-selectin in the plasma of the mutant mice (Fig. 10 *a*). Consistent with the result of the ELISA, Western blot analysis of the plasma using a polyclonal antibody against P-selectin revealed a fragment at ~100 kD whose level was significantly increased in ΔCT mice compared with wild-type mice (Fig. 10 *b*). Since the capture antibody used in the ELISA is a monoclonal antibody (RB40.34) against the lectin domain of P-selectin (Bosse and Vestweber, 1994), and because of the estimated molecular weight, we believe that the P-selectin fragment in the plasma detected by Western blot contains the NH_2-terminal 100-kD extracellular portion of P-selectin. Consistent with this possibility, the soluble P-selectin fragment in the plasma was not recognized by the anti-CT antibody in Western blot analysis (data not shown). In addition, treatment of wild-type mice with LPS or TNF-α induced a fourfold increase in the levels of soluble P-selectin in the plasma (Fig. 10 *c*), indicating that shedding of P-selectin into the plasma may represent a general mechanism of P-selectin downregulation after inflammatory responses.

Discussion

To evaluate in vivo the importance of regulated secretion of P-selectin, we have generated, by gene replacement, a mutant mouse that expresses P-selectin lacking the cytoplasmic domain. We have verified that the truncated form of P-selectin mRNA without the CT sequence is expressed by the mutant mice, and that neither platelet nor tissue-derived P-selectin from the mutant mice contains the cytoplasmic domain. Previous in vitro studies have shown that sorting signals within the CT of P-selectin are required for the compartmentalization of this molecule to storage granules in neuroendocrine or insulinoma cell lines (Disdier et al., 1992; Subramaniam et al., 1993), but, until now, it was not possible to evaluate the role of this domain in P-selectin storage in cell types normally expressing P-selectin, i.e., endothelial cells and platelets. To our surprise, P-selectin with a deleted cytoplasmic domain was stored in the α-granules of resting platelets as well as the wild-type molecule. Surface expression of the ΔCT–P-selectin on the mutant platelets was induced by thrombin in a similar fashion to wild-type platelets. On the other hand, the storage of P-selectin in lung endothelial cells was indeed heavily dependent on the presence of the cytoplasmic domain. Cultured lung endothelial cells isolated from the ΔCT mice displayed strong membrane expression of P-selectin as opposed to the Weibel-Palade body localization of the wild-type molecules (Fig. 7 *a*). Furthermore, calcium ionophore failed to upregulate leukocyte rolling in the mesenteric venules of the mutant mice, indicating an absence of a re-

leasable storage pool of P-selectin in mesentery endothelial cells in vivo. Therefore, it appears that both the lung and mesentery endothelial cells cannot efficiently target the ΔCT–P-selectin to their storage granules. The different fate of ΔCT–P-selectin in platelets and endothelial cells of these tissues may be due to differences in the two types of storages granules involved. In addition to proteins synthesized by megakaryocytes, α-granules also store proteins acquired from the surrounding plasma through receptor-dependent or independent endocytosis (George, 1990; Handagama et al., 1993). The packaging of plasma proteins into α-granules is another indication that the machinery for protein sorting in platelets differs from that of endothelial cells and neuroendocrine cells. Thus, it is possible that the requirements for protein sorting into platelet α-granules are less stringent than those for Weibel-Palade bodies of endothelial cells or other "more conventional" storage granules.

We have observed some heterogeneity among vascular beds of different tissues in sorting of ΔCT–P-selectin. Brain endothelial cells, which normally synthesize little P-selectin, when put in culture upregulate P-selectin expression which colocalizes with vWF in the Weibel-Palade bodies (Barkalow et al., 1996*a*). Although intense surface staining was still observed, some ΔCT–P-selectin was also localized in the Weibel-Palade bodies in brain endothelial cells of the mutant mice (Fig. 7 *b*). One hypothesis to explain the partial targeting of the mutant P-selectin is that sequence elements in P-selectin other than the cytoplasmic domain also contribute to the sorting of this molecule. The presence of the transmembrane domain of P-selectin in chimeric constructs was recently shown to enhance the efficiency of P-selectin sorting in insulinoma cells (Fleming et al., 1998). It is possible that the transmembrane domain of P-selectin plays a greater part in the sorting process in brain than in lung endothelial cells.

Previous in vitro studies have shown that the CT of P-selectin contains signals for its internalization from the plasma membrane (Subramaniam et al., 1993; Setiadi et al., 1995). Deletion of the cytoplasmic domain results in accumulation of P-selectin on the surface of transfected neuroendocrine cells (Norcott et al., 1996). Similarly, in the current study, we have observed intense surface staining of P-selectin on in vitro cultured ΔCT endothelial cells, which supports the notion that the internalization as well as the granular targeting of P-selectin is impaired in these cells. However, intravital microscopy studies did not reveal increased leukocyte–endothelium interactions in the ΔCT mice. Instead, we have detected a significant increase in soluble P-selectin in the plasma of the mutant mice. Western blot analysis revealed a 100-kD fragment of P-selectin in the plasma of both wild-type and mutant mice with its amount increased severalfold in the ΔCT plasma (Fig. 10 *b*). The molecular mass of this soluble P-selectin fragment is much smaller than that detected from the lysates of ΔCT platelets and it is recognized by an antibody against the lectin domain. These observations are most consistent with its being a cleavage product of ΔCT–P-selectin expressed on the surface of endothelial cells in vivo. We believe that the weaker P-selectin staining on the frozen sections of the mutant lung and heart compared with the wild type reflects the combined outcome of the impaired storage of P-selectin in the mutant endothelium and the proteolytic removal of the ΔCT–P-selectin constitutively delivered to the endothelial surface in vivo.

The fact that ΔCT–P-selectin is shed into the blood in vivo but remains on the surface of cultured endothelial cells in vitro suggests that certain signals or components required for the proteolytic cleavage of P-selectin in vivo are absent in the culture system in vitro. Recent studies have indicated that the same is true for shedding of P-selectin from platelets. Incubation of activated platelets in buffer in vitro results in little shedding of P-selectin whereas reinfusion of these platelets into mice causes rapid cleavage of P-selectin from the cell surface (Berger et al., 1998). Therefore, it appears that, at least for platelets, signals in addition to thrombin activation are required for shedding to occur; these may be provided by interactions with other cells in vivo. The precise proteolytic mechanisms responsible for cleavage of P-selectin are currently unknown. Recent studies have revealed that diverse cell surface proteins such as L-selectin, TNF-α, and IL-6 receptor are proteolytically cleaved by a system sensitive to metalloprotease inhibitors (Mullberg et al., 1994; Arribas et al., 1996; Walcheck et al., 1996). TNF-α converting enzyme (TACE), a metalloprotease that cleaves pro–TNF-α to generate the soluble form of TNF-α has been identified and cloned (Black et al., 1997; Moss et al., 1997). Cells with an inactivated TACE gene display defects in L-selectin shedding as well as TNF-α production (Black et al. 1997. A metalloproteinase-disintegrin releases TNF from cells. *Am. Soc. Cell Biol.*, 37th. 8:6*a*). It is possible that similar mechanisms may be used in the shedding of membrane P-selectin into the plasma. The molecular mass of the P-selectin fragment in plasma detected by Western blot together with the fact that it is recognized by an antibody to the lectin domain suggest that the cleavage may have occurred after CR6 in mouse P-selectin which corresponds to CR7 in the human molecule. Cloning of P-selectin provides evidence for a spacer of eight amino acids in this region, i.e., two CR repeats away from the transmembrane domain in both mouse and human P-selectin (Johnston et al., 1989; Sanders et al., 1992). Mutational analyses of the L-selectin cleavage site have shown that the distance between the cleavage site and the membrane rather than the primary sequence plays a critical role in directing proteolysis (Chen et al., 1995; Migaki et al., 1995). Therefore, the eight-amino acid spacer in P-selectin may be critical in enabling the access of proteases to the P-selectin molecules expressed on the plasma membrane. In addition, a recent study has suggested a functional link between the cytoplasmic tail of L-selectin and its membrane proximal cleavage region, i.e., mutations in the CT of L-selectin appear to affect the conformation of its extracellular cleavage site (Kahn et al., 1998). If such is also the case for P-selectin, one might predict that its susceptibility to proteolytic cleavage could be altered by the deletion of the CT.

The plasma of healthy humans and mice contains little soluble P-selectin as detected by ELISA (Dunlop et al., 1992) (Fig. 10 *a*). Increased concentrations of P-selectin in plasma have been reported in diseases such as thrombocytopenic purpura, atherosclerosis, and ischemic stroke (Chong et al., 1994; Blann et al., 1997; Frijns et al., 1997). Shedding of P-selectin appears also to happen in patholog-

ical situations in wild-type mice (Fig. 10 *c*). These observations suggest that in an inflammatory response where extensive endothelial surface expression of P-selectin is induced, shedding of this molecule may be a mechanism used in vivo, along with internalization, to downregulate the adhesiveness of the endothelium. Moreover, the shed-soluble P-selectin is likely to inhibit additional leukocyte adhesion and have a "calming" effect on the recruited neutrophils (Gamble et al., 1990; Wong et al., 1991), which again may limit excessive damage produced by inflammatory responses.

The adhesion of activated ΔCT platelets to HL-60 cells, the rolling of leukocytes on ΔCT endothelium, and the thioglycollate-induced neutrophil recruitment in the mutant mice all indicate that ΔCT–P-selectin is capable of mediating cell adhesion. This is consistent with previous in vitro studies which have shown that the lectin and EGF domain of P-selectin together, either expressed on membrane or in soluble form, are capable of binding to PSGL-1, the major ligand for P-selectin (Gibson et al., 1995; Mehta et al., 1997). However, despite the comparable levels of baseline leukocyte rolling between the wild-type and ΔCT mice, the lack of upregulation of leukocyte rolling immediately after stimulation (Fig. 8) is reflected in a reduced inflammatory response. The leukocyte influx in these mice 2 h after thioglycollate injection is only 30% of that in wild-type counterparts. This defect in leukocyte recruitment implicates the physiological importance of P-selectin storage in endothelial cells and suggests that the rapid translocation of P-selectin to cell surface is essential in the early stages of inflammation. The defect in ΔCT mice was less severe compared with P-selectin–deficient mice (Mayadas et al., 1993), supporting the functional capacity of ΔCT–P-selectin expressed constitutively in vivo. The correction of the defect at later time points could result from increased involvement of other adhesion molecules such as L- and E-selectin in both the wild-type as well as the ΔCT mice, as suggested by earlier studies (Arbonés et al., 1994; Labow et al., 1994), or a decreased influence of P-selectin storage in later stages of inflammatory responses. In this study we did not directly compare the affinity of the wild-type and the ΔCT–P-selectin molecules for leukocytes. It is possible that ΔCT–P-selectin binds less strongly than the wild type as seen in in vitro rolling studies (Setiadi et al., 1998).

In contrast to the results with P-selectin, deletion of 11 of the 17 amino acids in the L-selectin cytoplasmic domain completely abolishes both the binding of transfected pre-B cells to high endothelial venules and L-selectin–dependent rolling of these cells in vivo (Kansas et al., 1993). The cytoplasmic domain of L-selectin has also been shown to interact with α-actinin, a protein involved in linking adhesion molecules to the actin cytoskeleton, and with a calcium-binding protein calmodulin (Pavalko et al., 1995; Kahn et al., 1998). Interestingly, truncation of L-selectin cytoplasmic domain does not affect the localization of L-selectin to the microvillar projections (Pavalko et al., 1995). E-selectin cytoplasmic domain also becomes associated with components of the cytoskeleton as a consequence of leukocyte binding to its extracellular domain (Yoshida et al., 1996). Similar to P-selectin, E-selectin without cytoplasmic domain can support adhesion to HL-60 cells in vitro (Kansas and Pavalko, 1996; Yoshida et al., 1996). There is no evidence to date to indicate that P-selectin CT directly interacts with the cytoskeleton in platelets or endothelial cells. A recent study has indicated that the cytoplasmic domain of P-selectin mediates the clustering of the protein in clathrin-coated pits (Setiadi et al., 1998). In addition, the CT of P-selectin is reportedly phosphorylated on tyrosine, serine, threonine, and histidine residues after platelet activation (Fujimoto and McEver, 1993; Crovello et al., 1995), although the biological significance of these modifications is yet to be elucidated. The generation of the mice producing P-selectin without the cytoplasmic domain will allow study of the role of this domain in signaling events both in platelets and in endothelial cells obtained from these animals. In addition, this mouse constitutively producing high levels of soluble P-selectin will provide a vehicle for evaluating the possible anti-inflammatory effect of this P-selectin pool in various disease models.

The authors thank M. Berndt for providing antibodies for our study, J. Hartwig (Harvard Medical School, Boston, MA) for electron microscopy facilities, and D. Vestweber for mouse P-selectin cDNA. We thank W. Atkinson (Harvard Medical School) for his advice on preparation of lung endothelial cells. We also thank C. Moyna and S. Saffaripour (both from Center for Blood Research, Boston, MA) for their technical assistance, M. Ullman-Culleré (Massachusetts Institute of Technology, Cambridge, MA) and M. Economopoulos for mouse husbandry, and L. Cowan (both from Center for Blood Research) for editorial comments.

This work was supported by National Institute of Health (NIH) grants to D.D. Wagner (HL 53756), R.O. Hynes (HL 41484), and T.N. Mayadas (NS 33296). D.W. Hartwell was supported by an NIH Individual National Research Service Award (HL 09264) and P.S. Frenette was supported by a fellowship from the Medical Research Council of Canada. R.O. Hynes is an investigator of the Howard Hughes Medical Institute.

Received for publication 26 May 1998 and in revised form 10 September 1998.

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