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# Heparan Sulfate and Chondroitin Sulfate Proteoglycans Inhibit E-Selectin Binding to Endothelial Cells

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**Abstract** E-selectin is a cell adhesion molecule involved in the initial rolling and adhesion of leukocytes to the endothelium during inflammation. In addition, in vitro studies have suggested that an interaction between E-selectin and binding sites such as sialyl Lewis X-containing oligosaccharides on endothelial cells may be important for angiogenesis. In order to investigate the binding of E-selectin to endothelial cells, we developed an ELISA assay using chimeric E-selectin-Ig molecules and endothelial cells fixed on poly-L-lysine coated plates. Our results indicate that E-selectin-Ig binds to both bovine capillary endothelial cells and human dermal microvascular endothelial cells in a calcium-dependent and saturable manner. The binding is inhibited markedly by heparin and by syndecan-1 ectodomain, and moderately by chondroitin sulfate, but not by sialyl Lewis X-containing oligosaccharides. These results suggest that heparan sulfate and chondroitin sulfate proteoglycans on endothelial cells are potential ligands for E-selectin. J. Cell. Biochem. 80:522–531, 2001. 

**Key words:** selectins; microvascular; endothelium; syndecan-1; glycosaminoglycans

E-selectin, also known as endothelial-leukocyte adhesion molecule-1, is a member of the selectin family of adhesion molecules, which participates in leukocyte rolling and adhesion to the endothelium during inflammation [McEver, 1997; Lowe, 1997]. There are three members in the family; E-selectin, P-selectin, and L-selectin. E-selectin is expressed exclusively in the endothelium, P-selectin is expressed in platelets and in endothelium, and L-selectin is expressed in leukocytes [Bevilacqua and Nelson, 1993]. All three are transmembrane glycoproteins that contain an *N*-terminal C-type lectin domain, an epidermal growth factor-like domain, a variable number of complement-binding protein-like domains, a single transmembrane domain, and a short cytoplasmic tail. The structural similarities among the selectins confer similar carbohydrate binding properties to these adhesion molecules. The selectins bind with relatively low affinity to sialylated fucosylated oligosaccharides such as the sialyl Lewis X ($sLe^x$) (NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc) or sialyl Lewis A ($sLe^a$) (NeuAcα2,3Galβ1,3(Fucα1,4) GlcNAc) and related structures [Varki, 1994]. L- and P- selectin have been shown to interact with sulfated polysaccharides such as heparin, fucoidan, and dextran sulfate as well as inositol polyanions [Nelson et al., 1993a, Skinner et al., 1991, Cecconi et al., 1994]. However, E-selectin did not bind to heparin in these assays [Nelson et al., 1993a, 1993b].

Abbreviations used: PSGL-1, P-selectin glycoprotein ligand-1; GAG, glycosaminoglycans; BCE, bovine capillary endothelial cells; HDMEC, human dermal microvascular endothelial cells; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; FCS, fetal calf serum; CS, calf serum; PBS, phosphate-buffered saline; standard deviation, SD.

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Several glycoproteins with higher affinity for selectins have been identified. The best characterized selectin ligand to date is P-selectin glycoprotein ligand-1 (PSGL-1), a disulfide-linked homodimer present on leukocytes [McEver and Cummings, 1997]. PSGL-1 was purified from the human promyelocytic cell line HL-60 [Norgard et al., 1993] and its cDNA isolated from an HL-60 cell cDNA library by expression cloning [Sako et al., 1993]. Several laboratories have now demonstrated that the high affinity interaction of P-selectin with PSGL-1 requires both sialylated, fucosylated O-linked glycans and sulfated-tyrosine residues on PSGL-1 [Wilkins et al., 1995, 1996; Sako et al., 1995; Pouyani and Seed 1995; Moore et al., 1994]. E-selectin can also bind to PSGL-1 but the interaction does not appear to involve sulfated tyrosine residues and the binding affinity is reported to be 50-fold lower [Sako et al., 1995, Pouyani and Seed 1995, Moore et al., 1994]. A distinct ligand for E-selectin was purified from a murine myeloid cell line and found to be a homolog of a chicken cysteine-rich fibroblast growth factor receptor [Steegmaier et al., 1995]. This murine E-selectin ligand contains sialylated, fucosylated N-linked glycans that are required for recognition by murine E-selectin. E-selectin has also been shown to bind with high affinity to sialylated, fucosylated N-linked glycans expressed in human neutrophils [Patel et al., 1994]. L-selectin is expressed on leukocytes and binds to the mucin-like proteins GlyCAM-1 (sgp50), CD34 (sgp90) and MadCAM-1 present on murine lymph node high endothelial venules [Imai et al., 1993, Baumheter et al., 1993]. L-selectin has also been shown to bind sulfated derivatives of $sLe^x$ [Galustian et al., 1997] and heparan sulfate glycosaminoglycans (GAGs) derived from non-lymphoid endothelial cells [Norgard-Sumnicht et al., 1993]. Thus, the selectins can bind to an array of sialylated and sulfated oligosaccharides in in vitro assays. However, the structural elements recognized in vivo, as demonstrated by characterization of PSGL-1/P-selectin interactions, are likely to be complex.

In addition to its function in inflammation, several lines of experimental evidence suggest that E-selectin is involved in angiogenesis. First, E-selectin and $sLe^x$-containing oligosaccharides have been shown to be required for the formation of capillary blood vessel-like tubes in vitro [Nguyen et al., 1993]. Second, soluble E-selectin has been shown to induce neovascularization in rat cornea in vivo and to stimulate chemotaxis of endothelial cells in vitro [Koch et al., 1995]. Furthermore, antibodies directed against $sLe^x$ epitopes blocked the E-selectin-induced chemotaxis in vitro [Koch et al., 1995]. In addition, anti-E-selectin antibodies inhibited bFGF-induced neovascularization in mouse cornea in vivo (Yu et al., manuscript in preparation). These data suggest that endothelial cells express ligands for E-selectin and the interaction between E-selectin and its sialylated fucosylated ligands plays a role in angiogenesis. Finally, E-selectin is present in proliferating endothelial cells in non-inflammed angiogenic tissues and the level of expression correlates with the degree of angiogenesis [Kraling et al., 1996].

To identify and characterize E-selectin ligands on endothelial cells, we developed a modified ELISA assay to study the binding of E- and P-selectin to endothelial cells. Both E- and P-selectin bind to endothelial cells in a $Ca^{2+}$-dependent and saturable manner. The binding of E-selectin and P-selectin can be distinguished by other biochemical properties indicating that E- and P-selectins may recognize distinct ligands on endothelial cells. Although previous studies have not indicated an E-selectin–heparan sulfate proteoglycans interaction, we felt it was important to include GAGs in our analyses given the known promiscuity of the selectins and the importance of these GAGs regulating the functions of angiogenic factors. To our surprise, the binding of E-selectin was blocked by heparin, chondroitin sulfate and the heparan sulfate proteoglycan syndecan-1, but not by sialyl Lewis X-containing oligosaccharides, indicating that heparan sulfate and/or chondroitin sulfate proteoglycans on the endothelial cell surface may be ligands for E-selectin.

## METHODS

### Materials

Heparin from porcine mucosa was obtained from HEPAR Industries Inc. (Franklin, OH). Heparan sulfate from bovine kidney, chemically modified heparin (N-desulfated O-sulfated, N-sulfated O-desulfated and N, O-desulfated), chondroitin sulfate B (dermatan sulfate) from pig skin, and C (chondroitin 6-sulfate) from shark cartilage were from Seikagaku Kogyo

Co. Ltd (Tokyo, Japan); Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (1000 mg/l) was from JRH Biosciences (Lenexa, KS); fetal calf serum (FCS) and calf serum (CS) from Hyclone (Logan, UT); L-glutamine and GPS (0.29 mg/ml of L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin sulfate) from Irvine Scientific (Santa Ana, CA); endothelial basal media (EBM) from Clonetics (San Diego, CA); fungizone and NuSerum from Gibco; dibutyryl cAMP, hydrocortisone, Protein-A-Sepharose CL-4B, p-nitrophenyl-phosphate, and chloroquine from Sigma (St. Louis, MO); biotinylated goat-anti-human IgG Fc antibody and alkaline phosphatase-conjugated streptavidin from Caltag (Burlingame, CA); DEAE-Dextran and Sepharose CL-4B from, Pharmacia (Piscataway, NJ); BCA protein assay reagent from Pierce (Rockford, IL); Centricon-50 from, Amicon (Beverly, MA); human recombinant basic fibroblast growth factor (bFGF) was kindly provided by Scios Inc., (Mountain View, CA); $sLe^x$, $sLe^a$, and $SO_4$–$Le^x$ from Oxford Glycosystems (Rosedale, NY).

## Cell Culture

Bovine capillary endothelial (BCE) cells isolated from bovine adrenal cortex were cultured in DMEM supplemented with 10% heat-inactivated CS, GPS and 3 ng/ml bFGF [Folkman et al., 1979]. Bovine fibroblasts (BFB) were isolated by mild trypsin digestion of the connective tissue of bovine aorta and plated on tissue culture flasks in DMEM supplemented with 10% heat-inactivated CS and GPS. Bovine aorta endothelial cells (BAE) and NIH3T3 cells were cultured in the same media. Bovine retina pigment epithelial cells (BRPE) were cultured in DMEM supplemented with 10% heat-inactivated FCS, and GPS. Human dermal microvascular cells (HDMEC) were isolated from neonatal foreskin as described [Kraling and Bischoff, 1998] and grown in endothelial basal media (EBM131) supplemented with 10% heat-inactivated FCS, 400 U/ml penicillin, 400 μg/ml streptomycin, 2 mM L-glutamine, and 2 ng/ml bFGF.

## Construction and Expression of Chimeric Selectin-Ig Molecules

A bovine E-sel-Ig chimeric protein that contains the first 404 amino acids of the *N*-terminus of bovine E-selectin was prepared as described [Aruffo et al., 1990]. Human E-sel-Ig, and human P-sel-Ig were provided by Brystol-Meyers Squibb [Walz et al., 1990, Aruffo et al., 1991, Hollenbaugh et al., 1992]. As shown in Figure 1, each chimeric molecule contains a selectin lectin domain, an epidermal growth factor domain and four (bovine E-sel-Ig), six (human E-sel-Ig), or two (human P-sel-Ig) complement regulatory protein (CR) domains fused to the hinge domain (CH2 and CH3 regions) of human $IgG_1$. Recombinant chimeric proteins

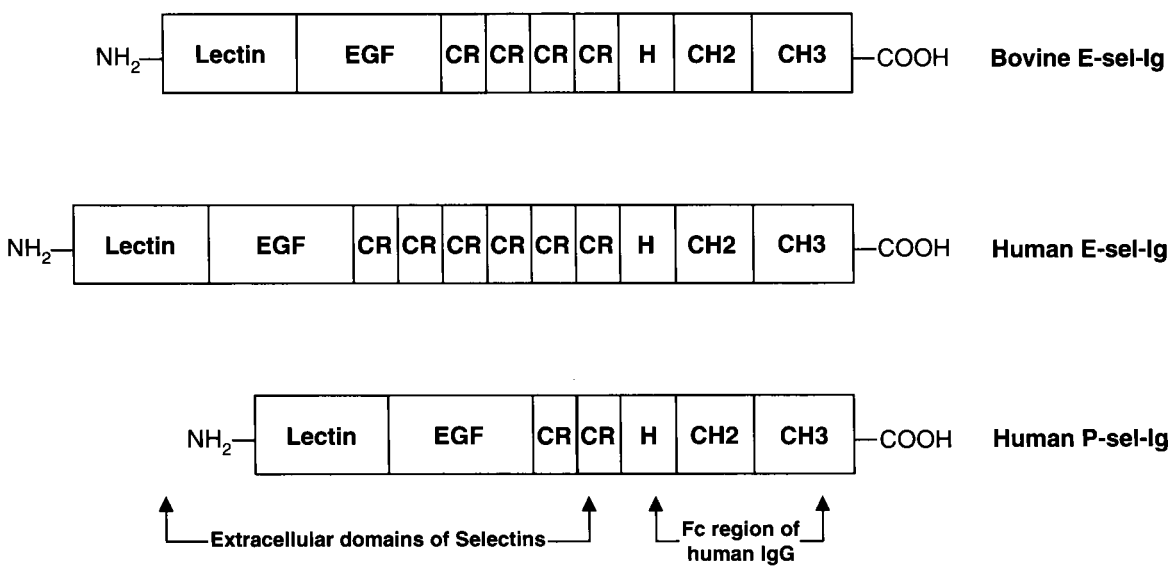


**Fig. 1.** A diagram showing the structures of recombinant chimeric E-selectin-Ig and P-selectin-Ig molecules.

that contain the extracellular domains of cell adhesion molecules or receptors fused to the Fc domain of the immunoglobulin heavy chain are widely used because of the ease of purification and detection using immunological reagents [Hollenbaugh et al., 1992].

### Purification of Bovine E-Selectin-Ig

The bovine E-selectin-Ig cDNA-containing plasmid was transfected into COS cells as described [Seed and Aruffo, 1987]. Serum-free media from transfected COS cells were harvested and centrifuged at 3400 rpm at 4°C for 30 min to remove cells and debris. The pH of the supernatant was adjusted by adding 1/10 (v/v) of 10 × PBS (phosphate-buffered-saline) before loading onto a 1 ml Protein-A sepharose CL-4B column equilibrated in PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (equilibration buffer). Bound E-sel-Ig chimeric proteins were eluted using 6 × 1 ml of 4 M imidazole, pH 8.0, 1 mM $CaCl_2$, 1 mM $MgCl_2$. Protein elution was monitored by spectrophotometry at 280 nm. Fractions containing protein were pooled, and dialyzed against the equilibration buffer made with non-pyrogenic water and concentrated in Centricon-50 presoaked with 5% Tween-20. The concentration of E-sel-Ig was determined using BCA protein assay with bovine serum albumin (BSA) as a standard. Human E- and P-sel-Ig, purified in a similar manner, were kindly provided by Drs. Mark Cunningham, Diane Hollenbough and Richard Darveau (Bristol-Meyers Squibb Pharmaceutical).

### Preparation of Syndecan-1 Ectodomain From Mammary Gland Epithelial Cells

Syndecan-1 ectodomain was purified to homogeneity from the conditioned medium of cultured NMuMG mouse mammary epithelial cells as previously described [Jalkanen et al., 1985]. Briefly, conditioned medium was freed of exogenous proteins by DEAE-anion exchange column chromatography at pH 4.5, and the proteoglycans isolated by isopycnic centrifugation in CsCl. Finally, purified syndecan-1 ectodomain was obtained by immunoaffinity column chromatography with monoclonal antibody 281-2 against mouse syndecan-1. Amino acid and sugar analyses established the concentration of the syndecan-1 ectodomain, and the values confirmed for each ectodomain preparation by radioimmunoassay for the ectodomain core protein [Kato et al., 1998].

### Binding of Soluble E-Sel-Ig to Endothelial Cells

BCE or HDMEC cells were removed from dishes by gentle scraping in PBS/5 mM EDTA. The cells were centrifuged, counted, and plated on poly-L-lysine coated 96-well plates (100 μl/well) at a concentration of $8 \times 10^5$ cells/ml as described [Heusser et al., 1981]. The plate was centrifuged at 800 rpm for 10 min at 4°C and cells were fixed in 0.25% glutaraldehyde in PBS for 10 min at 4°C. After washing with PBS, unoccupied sites were blocked with TBS/BSA (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 1% BSA) at 4°C overnight. E-sel-Ig or P-sel-Ig at indicated amounts was incubated with fixed BCE cells at 50 μl per well for 1 h at room temperature in the presence of TBS/BSA/1 mM $CaCl_2$/1 mM $MgCl_2$ or TBS/BSA/10 mM EDTA. Unbound molecules were removed by washing the wells twice in TBS/BSA/1 mM $CaCl_2$/1 mM $MgCl_2$ or TBS/BSA/10 mM EDTA; and once in TBS/BSA. Bound E-sel-Ig or P-sel-Ig molecules were detected by incubation with biotinylated goat-anti-human IgG Fc antibodies and alkaline phosphatase conjugated streptavidin diluted 1:1000 in TBS/BSA. After adding 100 μl per well of the substrate *p*-nitrophenyl phosphate dissolved in the substrate buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), absorbance was read at 410 nm in a Dynatech ELISA plate reader MR5000. $Ca^{2+}$-dependent binding was calculated by subtracting the binding obtained in the presence of EDTA from that obtained in the presence of 1 mM $CaCl_2$/1 mM $MgCl_2$. Each data point was performed in quadruplicate unless otherwise noted. Results were plotted as mean ± standard deviation (SD) using the Sigmaplot.

## RESULTS

### E-Sel-Ig Binding to Endothelial Cells is $Ca^{2+}$-Dependent and Saturable

The presence of potential E-selectin ligands on endothelial cells was assessed by incubation of soluble recombinant E-sel-Ig molecules with BCE or HDMEC cells that had been gently scraped from tissue culture dishes and fixed on poly-L-lysine coated 96-well plates. Human P-sel-Ig was analyzed in parallel since P-selectin has similar carbohydrate-binding properties. HL-60 cells were also analyzed in parallel as a positive control since E- and P-selectin are

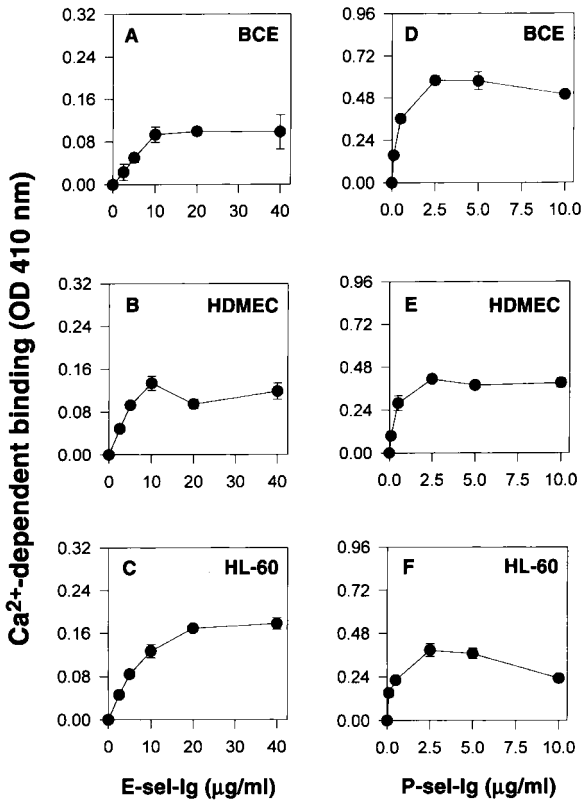


**Fig. 2.** $Ca^{2+}$-dependent binding of E-sel-Ig and P-sel-Ig to BCE, HDMEC and HL-60 cells. BCE cells (Panels A and D) or HDMEC cells (Panels B and E) were gently scraped from culture dishes and fixed onto poly-L-lysine-coated 96-well culture plates at $8 \times 10^4$ cells/well. HL-60 cells (Panels C and F) were washed in PBS and fixed onto poly-L-lysine-coated 96-well culture plates. Increasing amount of human E-sel-Ig (panels A, B, and C) or P-sel-Ig (panels D, E, and F) were applied to fixed cells. Bound selectin-Ig molecules were detected, and the $Ca^{2+}$-dependent binding of E-sel-Ig or P-sel-Ig was calculated as described under Materials and Methods. A representative experiment from among three is shown.

known to bind this human promyelocytic cell line.

E-sel-Ig bound to bovine and human microvascular endothelial cells and to HL-60 cells in a $Ca^{2+}$-dependent and saturable manner (Fig. 2). The $Ca^{2+}$-dependent binding of E-sel-Ig to BCE cells (Panel A) and to HDMEC cells (Panel B) was saturated at 10 μg/ml. This corresponds to an apparent dissociation constant of approximately 43 nM for E-sel-Ig. Similar binding to HL-60 cells was observed (Panel C). This binding affinity is comparable to the dissociation constant of 70 nM for the binding of soluble P-selectin monomers to neutrophils and HL-60 cells [Ushiyama et al., 1993] and to that reported for soluble human E-selectin binding to HL-60 cells [Burrows et al., 1995]. The binding of P-sel-Ig to BCE cells (Panel D), HDMEC cells (Panel E), and HL-60 cells (Panel F) was also $Ca^{2+}$-dependent and saturable. The binding plateaued at 2.5 μg/ml for all three cell types suggesting that the recombinant P-sel-Ig binds with higher affinity than E-sel-Ig. This higher affinity interaction between P-selectin and the ligands displayed by the cells may be due to differences in the degree to which the recombinant molecules have folded into their native states. As described in Materials and Methods, the recombinant human E-sel-Ig molecules have the same number of CR domains as their parent polypeptides while the recombinant P-sel-Ig has only two of the nine CR domains in human P-selectin.

### Binding of E-Sel-Ig to BCE Cells can be Abrogated by Pre-Adsorption with Protein A-Sepharose or HL-60 Cells

The specificity of E-selectin binding to BCE cells was tested by pre-adsorption of E-sel-Ig with Protein A-Sepharose, Sepharose, or HL-60 cells. The pre-adsorption was done by incubating 5 μg of E-sel-Ig with 100 μl of Protein A-Sepharose, or Sepharose, or with $5 \times 10^6$ HL-60 cells in 600 μl of PBS plus 1% BSA at 4°C for 1 h, followed by centrifuging at 14,000 rpm in a microfuge for 30 s. The supernatant was added to BCE cells that were fixed on 96-well plates, and analyzed for binding activity. As shown in Figure 3, pre-adsorption with Protein A-Sepharose or with HL-60 cells abolished the binding of E-sel-Ig to BCE cells while Sepharose did not. The depletion of binding activity by HL-60 cells indicates that the E-sel-Ig binding to endothelial cells is functionally similar to the binding to HL-60 cells.

### Binding of E-Sel-Ig and P-Sel-Ig to Different Cell Types

To determine if E-selectin binds to other cell types in addition to endothelial cells and HL-60 cells, a number of different types of cultured cells was analyzed. Cells were scraped and fixed onto poly-L-lysine coated 96-well plates and assayed as described in Experimental Procedures. In Figure 4A, comparable levels of $Ca^{2+}$-dependent binding by E-sel-Ig to three endothelial cell lines, BCE, BAE, and HDMEC cells, were observed while very low levels of $Ca^{2+}$-dependent binding of E-sel-Ig to three

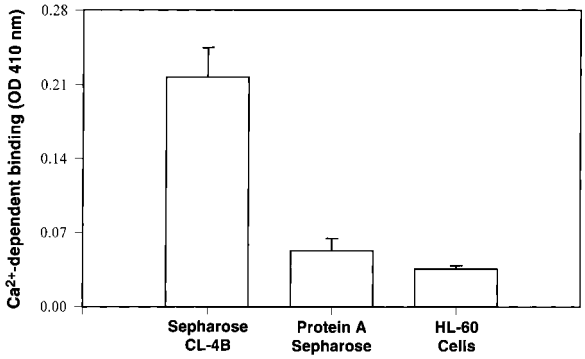


**Fig. 3.** The binding of human E-sel-Ig to BCE cells can be abrogated by pre-incubation with Protein A-Sepharose or HL-60 cells. 5 μg of human E-sel-Ig was incubated with 100 μl of BSA-blocked Protein A-Sepharose, Sepharose-CL-4B or $5 \times 10^6$/100 μl HL-60 cells at 4°C for 1 h with constant rotation. The mixture was then microfuged at maximum speed for 30 s to remove sepharose beads or cells. The supernatant was applied to BCE cells fixed on poly-L-lysine-coated plates and analyzed as described in Materials and Methods. A representative experiment from among three is shown.

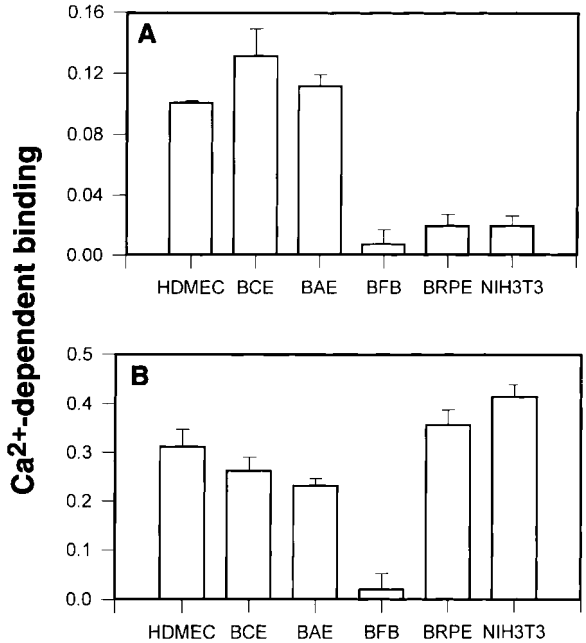


**Fig. 4.** Binding of E-sel-Ig and P-sel-Ig to different cell types. Cells were grown as described in Materials and Methods, scraped and fixed onto poly-L-lysine coated 96-well culture plates at $8 \times 10^4$ cells/well. BCE and BAE cells were fixed for 10 min at 4°C in 0.25% glutaraldehyde while other cell types were fixed for 20 min at 4°C. Human E-sel-Ig at 10 μg/ml (Panel A) or P-sel-Ig (Panel B) at 0.5 μg/ml were then applied to the cells and binding measured as described in Materials and Methods. A representative experiment from among three is shown. HDMEC: human dermal microvascular endothelial cells, BCE: bovine capillary endothelial cells, BAE: bovine aorta endothelial cells, BFB: bovine fibroblast cells, BRPE: bovine retina pigment epithelial cells, NIH3T3: SV40 transformed mouse fibroblast cells.

non-endothelial cell lines, BFB, BRPE, and NIH3T3 cells, were detected. In contrast, P-sel-Ig exhibited $Ca^{2+}$-dependent binding to all cell types tested except BFB cells (Fig. 4B) indicating that its binding sites are more ubiquitous than those of E-selectin.

### Differential Effects of Trypsinization on E-Sel-Ig and P-Sel-Ig Binding to Endothelial Cells

To assess the effects of different cell-harvesting methods on E- and P-selectin-Ig binding activity, endothelial cells were harvested either by gently scraping from the cell culture dish in PBS/5 mM EDTA or by mild trypsin/EDTA treatment (5 min at room temperature) to prepare a single cell suspension from the endothelial monolayer. E-sel-Ig exhibited a two-fold higher binding to BCE cells prepared by mild trypsinization (Fig. 5, solid bars) compared to those prepared by scraping (Fig. 5, hatched bars). In contrast, lower binding of P-sel-Ig was detected in trypsinized cells compared to scraped cells (Fig. 5). This finding suggests that more E-selectin ligands are exposed on trypsinized cells than that on scraped cells. Alternatively, P-selectin ligands may be removed from cell surfaces by trypsinization. Another possibility is that non-specific damage to the cell membrane caused by scraping cells adversely affects E-selectin binding sites but not P-selectin binding sites. In either case, the data suggest that E- and P-selectin interact with different endothelial binding sites.

### Heparin, Chondroitin Sulfate and Syndecan-1 Ectodomain Inhibit the Binding of E-Sel-Ig to Endothelial Cells

We assessed the effect of heparin on E-sel-Ig binding to BCE cells. Pre-incubation of 10 μg/ml of E-sel-Ig with increasing concentrations of heparin from 1 to 500 μg/ml, inhibited the binding of E-sel-Ig to BCE cells (Fig. 6A). Low concentrations of heparin (1 μg/ml) inhibited E-sel-Ig binding by 70% while 500 μg/ml heparin completely abolished the binding of E-sel-Ig to BCE cells (Fig. 6A). Similar results were obtained when heparin was added to the BCE cells together with E-sel-Ig without pre-incubation (data not shown). Pre-incubation of up to 100 μg/ml of heparin with 0.5 μg/ml of P-sel-Ig only partially (up to 50%) inhibited its

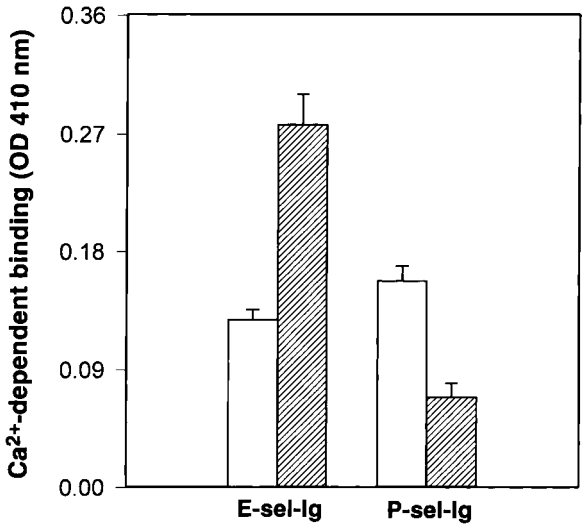


**Fig. 5.** Effect of trypsin digestion on binding of E-sel-Ig and P-sel-Ig to endothelial cells. Cells were removed from culture dishes by either gentle scraping in PBS, 1 mM EDTA (open bars) or by a mild trypsin digestion (hatched bars), washed once in PBS, plated and fixed on poly-L-lysine coated 96-well culture plates at $8 \times 10^4$ cells/well. 10 μg/ml of human E-sel-Ig or 0.5 μg/ml of human P-sel-Ig was applied to fixed cells, and binding to the cells measured as described in Materials and Methods. A representative experiment from among three experiments is shown.

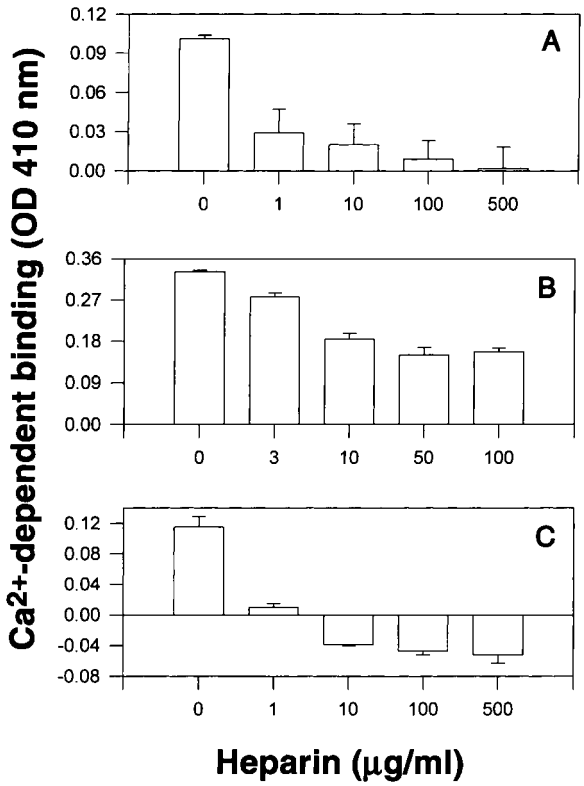


**Fig. 6.** Effect of heparin on the $Ca^{2+}$-dependent binding of E-sel-Ig and P-sel-Ig to BCE cells. In Panel A: E-sel-Ig (10 ug/ml) was pre-incubated with increasing amounts of heparin (HEPAR Industries Inc.) at 37°C for 1 h. The mixture was then added to fixed BCE cells and the binding of E-sel-Ig was assayed as described in the Materials and Methods. In Panel B: P-sel-Ig (0.5 ug/ml) was pre-incubated with increasing amounts of heparin at 37°C for 1 h, applied to fixed BCE cells and binding measured as in Panel A. In Panel C: E-sel-Ig (10 ug/ml) was pre-incubated with increasing amounts of heparin at 37°C for 1 h, applied to fixed HL-60 cells and binding measured as in Panel A. A representative experiment from among three is shown.

binding to BCE cells (Fig. 6B), indicating that P-selectin binding sites on BCE cells may be different from those of E-selectin. A low level of heparin (1 μg/ml) also strongly inhibited E-sel-Ig binding to HL-60 cells (Fig. 6C).

To define the carbohydrate components for E-selectin binding to endothelial cells, we tested a series of defined GAG chains and the purified syndecan-1 ectodomain as well as sialyl Lewis X oligosaccharides for inhibition of E-sel-Ig binding to endothelial cells. Syndecan-1 is a cell surface proteoglycan that contains both heparan sulfate and chondroitin sulfate GAG chains [Bernfield, 1999]. As shown in Figure 7, 4 μg/ml of syndecan-1 ectodomain as core protein, corresponding to 20 μg/ml heparan sulfate, and 20 μg/ml of heparin or N-desulfated O-sulfated-heparin inhibited the binding of E-sel-Ig to BCE cells by more than 85%. N-sulfated O-desulfated heparin at 20 μg/ml only caused about 30% inhibition while N, O-desulfated heparin at 20 μg/ml did not cause any significant inhibition. This result indicates that heparan sulfate containing proteoglycans such as syndecan-1 are important for E-selectin binding to BCE cells. Further, O-sulfation, but not N-sulfation on heparan sulfate/heparin sugar back bone is involved in the binding. Inhibition caused by chondroitin sulfate B and C at 20 μg/ml were about 60 and 50%, respectively, suggesting that chondroitin sulfate proteoglycans expressed on BCE are also involved in E-selectin binding to BCE cells. Alternatively, these chondroitin sulfates may partially inhibit E-selectin binding to heparan sulfate proteoglycans on BCE cells. The binding appeared not due to simple negative charge from sulfate residues on the GAGs because sulfated $Le^x$ which contains an O-sulfated galactose residue, had no effect on binding when tested at 20–400 μg/ml (Fig. 7 and data not shown). Hyaluronic acid which lacks sulfated sugars had no significant effect on E-sel-Ig binding to BCE cells (Fig. 7). $SLe^x$ and $sLe^a$ did

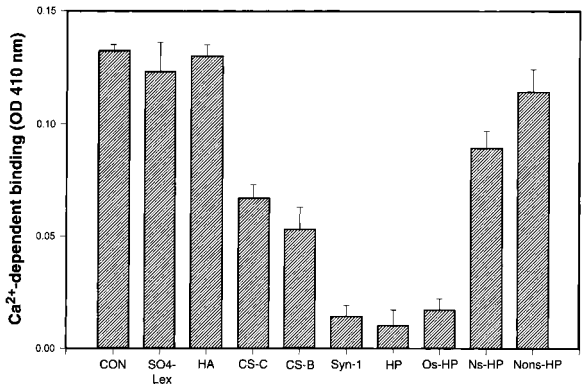


**Fig. 7.** Effects of GAGs on the $Ca^{2+}$-dependent binding of E-sel-Ig to BCE cells. Bovine E-sel-Ig (20 μg/ml) was pre-incubated with different GAG chains (4 μg/ml Syn-1 ectodomain, 20 μg/ml GAG chains, heparin, hyaluronic acid and sulfated $Le^x$) at 37°C for 30 min. The mixture was then added to fixed BCE cells and the binding of E-sel-Ig was determined as described in Materials and Methods. Results are shown as the mean ± standard error of triplicate samples from three independent experiments. CON (control): E-sel-Ig was pre-incubated in TBS/BSA. SO4-$Le^x$: sulfated $Lewis^x$, HA: Hyduronic acid, CS-C: Chondroitin sulfate C, CS-B: Chondroitin sulfate B, Syn-1: Syndecan-1 ectodomain, HP: heparin, Os-HP: N-desulfated O-sulfated heparin, Ns-HP: N-sulfated O-desulfated heparin, Nons-HP: N, O-desulfated heparin.

not inhibit binding at concentrations as high as 400 μg/ml (data not shown). Similar results were also obtained when HDMEC cells were analyzed in parallel (data not shown).

Activation of cultured human umbilical vein endothelial cells with TNF-α results in increased GAG levels in the medium and consequently decreased cell surface GAGs [Klein et al., 1992]. In another study, TNF-α downregulated syndecan-1 mRNA and protein in endothelial cells [Kainulainen et al., 1996]. Interestingly, incubation of BCE cells with TNF-α for 6 h resulted in decreased binding of E-selectin to BCE cells (data not shown). This result is consistent with our findings that cell surface heparan sulfate proteoglycans are potential E-selectin ligands on endothelial cells.

## DISCUSSION

We have demonstrated for the first time that E- and P-selectin bind to endothelial cells in a $Ca^{2+}$-dependent and saturable manner. The E-sel-Ig binding can be distinguished from P-sel-Ig binding by several biochemical properties such as sensitivity to trypsin and inhibition by heparin. The most striking property was the ability of heparin to inhibit E-sel-Ig binding to endothelial cells while $sLe^x$ oligosaccharides failed to inhibit the binding. Selectins have been shown to interact with sulfated oligosaccharides [Mulligan et al., 1998] and inositol polyanions [Cecconi et al., 1994]. Others have shown that heparin inhibits P- and L-selectin but not E-selectin binding to $sLe^x$-BSA [Nelson et al., 1993a]. Our analysis showed that E-sel-Ig binding to endothelial cells was inhibited effectively by N-desulfated O-sulfated heparin, indicating that O-sulfation is required for binding. Furthermore, the ectodomain of syndecan-1, a cell surface proteoglycan containing both heparan sulfate chain and chondroitin sulfate chains, strongly inhibited the binding of E-sel-Ig to endothelial cells. Of note, both chondroitin sulfate B (dermatan sulfate) and chondroitin sulfate C (chondroitin 6-sulfate) partially inhibited E-sel-Ig binding to endothelial cells. These results suggest that cell surface heparan sulfate and/or chondroitin sulfate proteoglycans, but not $sLe^x$, are ligands for E-selectin on endothelial cells. Interestingly, the binding site of E-sel-Ig on endothelial cells appeared to be more exposed by trypsinization (Fig. 5), which likely removes syndecan-1 from the cell surface [Jalkanen, 1987]. Thus, heparan sulfate proteoglycans other than syndecan-1 may be involved in E-selectin binding.

Previous results on the characterization of selectin ligands have revealed three important aspects. First, selectin ligands on cells are complex. Indeed, PSGL-1, the best characterized selectin ligand, requires both sialylated, fucosylated O-linked glycans and sulfated-tyrosine residues for high affinity interaction with P-selectin [Moore et al., 1991]. ESL-1 and Gly-CAM-1 also contain sialylated, fucosylated structures [Steegmaier et al., 1995, Imai et al., 1993]. Second, selectins have overlapping specificities to ligands in in vitro assays. For example, all three selectins can bind to PSGL-1. Sulfated tyrosine on PSGL-1 is required for its binding to P-selectin and L-selectin, but is not required for binding to E-selectin [Pouyani and Seed, 1995]. Differential effects of heparin on E- and P-sel-Ig binding to endothelial cells are consistent with these data. Third, selectin ligands identified by in vitro assays provide important clues for studying the in vivo ligands.

Although $sLe^x$ at high concentration can block the binding of selectins to carbohydrate

structures, the binding of $^{125}$I-labeled purified P-selectin to human neutrophils could not be blocked by sLe$^x$-containing neo-glycoproteins [Nelson et al., 1993b]. In addition, some sLe$^x$-containing glycoproteins such as L-selectin were not detected by $^{125}$I-labeled purified P-selectin in Western blots or by E-sel-Ig from detergent extracts of metabolically labeled neutrophils and 32D c1 3 cells [Levinovitz et al., 1993]. These results also illustrate the complexity of selectin ligands. The binding of 10 μg/ml of E-sel-Ig to endothelial cells was strongly inhibited by pre-incubation of E-sel-Ig with HL-60 cells (Fig. 3) but not by adding 400 μg/ml of sLe$^x$ or sLe$^a$ (data not shown) or sulfated Le$^x$ (Fig. 7). Therefore, our results agree well with the notion that sLe$^{x/a}$ structures are biologically relevant only when presented in the context of intact glycoprotein ligand structures. Other structures such as sulfated GAG may also be involved in high affinity binding of selectins to their physiological ligands.

Crude heparin from porcine intestinal mucosa has been reported to inhibit the binding of L- and P-sel-Ig, but not E-sel-Ig to immobilized BSA-sLe$^x$ (5). Heparin and fucoidan were also found to inhibit P-sel-Ig but not E-sel-Ig binding to immobilized BSA-sLe$^a$ (43). Our results indicate that heparin can inhibit both E-and P-sel-Ig binding to endothelial cells. This may not be contradictory to previous results since E-selectin ligands presented in the context of the microvascular endothelial cell plasma membrane in our cell-based assay may be far more complex than BSA-sLe$^{x/a}$.

Although PSGL-1 also binds E-selectin, it does not appear to be the E-selectin ligand on endothelial cells. First, E-selectin binding to endothelial cells could not be blocked by pre-incubation of endothelial cells with anti-PSGL-1 antibodies (data not shown). Second, PSGL-1 could not be detected in HDMECs by RT-PCR or Northern blot (data not shown).

Angiogenesis involves endothelial cell migration, proliferation, and tube formation. E-selectin has been shown to be associated with each of these steps. However, the precise role of E-selectin in angiogenesis has remained elusive. Since endothelial cells must align and adhere to each other to form tubes, the interaction between E-selectin and its cell surface proteoglycan ligands may contribute to the endothelial cell rearrangements required for the formation of a new blood vessel. The mechanisms involved are likely very complex, but further attention to E-selectin and endothelial cell surface proteoglycans is warranted.

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