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REGULATION OF P-SELECTIN BY TUMOR NECROSIS FACTOR-α[1]

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SUMMARY: The levels of P-selectin mRNA and polypeptide were analyzed in bovine capillary cells treated with or without the cytokine tumor necrosis factor-α. The 3 kb P-selectin mRNA was upregulated three- to five-fold in cytokine-stimulated cells. The increase in mRNA correlated with a dramatic but short-lived increase in P-selectin polypeptide as determined by metabolic-labeling and immunoadsorption. These data confirm earlier studies on mouse P-selectin expressed in a mouse endothelioma cell line and further indicate that P-selectin function can be regulated not only by rapid translocation to the cell surface but also by cytokine-stimulation of P-selectin biosynthesis.

P-selectin (GMP-140, PADGEM, CD62) functions as an adhesion receptor for leukocytes by initiating rolling of these cells along the endothelium at sites of inflammation (For review, see Refs. 1 and 2). P-selectin function is regulated in part by its sequestration within the α-granules of platelets and Weibel-Palade bodies of endothelial cells. Within minutes after activation of cells with agonists such as thrombin, P-selectin is transported to the cell surface where it can bind leukocytes. A definitive role for P-selectin *in vivo* has come from genetically-engineered P-selectin deficient mice that exhibit dramatically reduced leukocyte rolling and delayed extravasation (3). P-Selectin binds to carbohydrate ligands containing the tetrasaccharides sialyl-Lewis-X (Siaα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-R3) or sialyl-Lewis-A (Siaα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-R3) as well as other related compounds (For review, see Ref. 4). Recently, a high-affinity glycoprotein ligand for P-selectin that contains sialylated, fucosylated O-linked poly-N-acetyllactosamine has been isolated from human neutrophils (5).

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The abbreviations used are: TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; BCE, bovine capillary endothelial; GAPDH, glyceraldehyde-3-phosphate dehydrogenase SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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E-selectin, another member of the selectin family, was identified because of its inducible expression in response to TNF-α, interleukin-1 and LPS, agents that cause specific and reversible changes in endothelial cell surface properties. While P-selectin is expressed constitutively in endothelial cells, E-selectin mRNA and polypeptide expression is induced 2-8 hours after exposure to cytokines (1,2). The kinetics of E- and P-selectin expression at the cell surface is thought to regulate their participation in leukocyte adhesion. Recently, murine P-selectin mRNA and polypeptide have been shown to be upregulated by TNF-α in a murine embryonic endothelioma cell line (6) and in murine lung tissue after LPS stimulation (3,7). In this study, we demonstrate that P-selectin mRNA and polypeptide are upregulated by TNF-α and LPS in non-transformed bovine capillary endothelial cells. The cytokine-induced expression of P-selectin may allow it to function for prolonged periods of time in inflammatory settings.

EXPERIMENTAL PROCEDURES

Materials - Proteinase K, human recombinant TNF-α, random-primer cDNA labeling kit, and P^{1}5'-(7-methyl)-guanosine-P$_3$-5'-guanosine triphosphate, Boehringer Mannheim; oligo-d(T)-cellulose Type I, Collaborative Research; nitrocellulose BA85, Schleicher and Schuell; 5'α-[^{32}P]dCTP (3000 Ci/mmole), NEN-Dupont; [^{35}S]cysteine (> 1000 Ci/mmole), ICN; Protein A-Sepharose Cl-4B, Sepharose Cl-4B, leupeptin, aprotinin, LPS, and cyclohexamide, Sigma. Basic fibroblast growth factor was kindly provided by Scios-Nova, Inc., Mountain View, CA.

Cell culture - BCE cells were grown on gelatin-coated dishes in Dulbecco's modified Eagles medium, 10% heat-inactivated calf serum, 2mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 ng/ml basic fibroblast growth factor in a 10% CO_2 incubator as described (8). Cells were split 1:6 every 6-10 days and used between passage 10-14. TNF-α, LPS, and cyclohexamide were added directly to cells without changing the media.

Isolation of RNA and Northern blot analysis - RNA was isolated by lysing BCE cells in Proteinase K and SDS and then adsorbing polyA-containing RNAs to oligo-dT-cellulose (9). RNA preparations were fractionated by 1% agarose-formaldehyde gel electrophoresis and transfered to nitrocellulose. A 2kb cDNA fragment of bovine P-selectin was excised from the pBluescript plasmid containing the bovine P-selectin cDNA (10) with *BamHI* and *Hind*III and labeled with [α-^{32}P]dCTP using a random primer labeling kit. To detect bovine E-selectin transcripts, a 1.7kb *Kpn*I fragment of bovine E-selectin cDNA (11) was radiolabeled by the same method. [^{32}P]-labeled rat GAPDH cDNA was used as an internal standard (11). Hybridizations were carried out in 50% formamide, 50mM $NaPO_4$, pH 7.4, 750mM NaCl, 1mM EDTA, 5X Denhardt's, 0.1% SDS, 100μg/ml poly(A)$^+$, and 100μg/ml heat-denatured salmon sperm DNA at 42°C for 16 hours, washed 2 times for 5 minutes in 30mM sodium citrate, 300mM NaCl, 0.1% SDS, pH 7 at room temperature and then 4 x 15 minutes in 3mM sodium citrate, 30mM NaCl, 0.1% SDS, pH 7 at 50°C. The signals were visualized and quantitated by phosphor imager analysis. The internal control, GAPDH, was used to control for variations in RNA loading and transfer. [^{32}P]-labeled cDNA probes were removed from the nitrocellulose by incubating the membrane for 1 hour at 65°C in 60% formamide, 75mM sodium citrate, 750mM NaCl, 0.1% SDS. Membranes were exposed to a phosphor screen for 24 hours to check for complete removal of [^{32}P] signal.

In vitro transcription to prepare bovine P and E-selectin RNA standards - The pBluescript-SK plasmids containing bovine P-selectin and bovine E-selectin were linearized with *Xba*I and *BamHI* respectively. RNA transcripts were synthesized from these templates using T7 RNA polymerase in the presence of P^{1}5'-(7-methyl)-guanosine-P$_3$-5'-guanosinetriphosphate. Synthetic transcripts were run to verify that [^{32}P]-labeled bovine E- and P-selectin cDNA probes

hybridized only with their respective transcripts under the conditions used for Northern blot analyses (see Fig. 1).

Bovine P-selectin antiserum - A rabbit polyclonal antibody against bovine P-selectin was described previously (10). Briefly, a 511 bp fragment of the bovine P-selectin cDNA (coding for amino acids 57 to 227), was ligated into the *Sma*I site of the bacterial expression vector pGEX1, which contains the glutathione S-transferase (GST) gene. The 46 kDa insoluble GST/P-selectin fusion protein was solubilized with 8M urea and used to immunize rabbits.

Metabolic-labeling of BCE cells - Cells were trypsinized and split 1:6 and grown for 7 days in 60-mm dishes before metabolic-labeling. Monolayers were rinsed twice with PBS, incubated for 30 minutes at 37°C in growth media devoid of cysteine, and then pulse-labeled for 1 hour in 1.0 ml of cysteine-free media containing [^{35}S]cysteine (200 μCi/ml). At the end of the labeling period, the radioactive media was removed and the cell monolayers were either washed twice with ice-cold PBS (pulse) or further incubated in 4 ml of growth media at 37°C for the times indicated (chase).

Immunoadsorption of P-selectin - All steps were carried out on ice or at 4°C. BCE cells were solubilized with PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.005% SDS, 1 mM EDTA, 0.1 mM leupeptin, and 12.5 μg/ml aprotinin (0.5 ml per 60-mm dish). The detergent extract was precleared by incubating first with 250 μl of a 1:1 suspension of Sepharose Cl-4B in PBS for 30 min, centrifuging at 16,000 x g for 10 min, incubating with 80 μl of Protein A-Sepharose for 30 min and centrifuging again. The supernatant was then adjusted to 2.5 mg/ml bovine serum albumin. Half of the sample was incubated with preimmune serum (7 μl) and the other half with anti-P-selectin serum (7 μl) for 16 h on ice. Antigen-antibody complexes were adsorbed with 35 μl of Protein-A-Sepharose for 30 min and then sedimented, washed, and solubilized for SDS-PAGE analysis as described (12). The SDS-gel was fixed in 50% methanol, 10% acetic acid, vacuum dried, and bands were visualized by phosphorimager analysis.

RESULTS AND DISCUSSION

The expression of P- and E-selectin mRNAs was examined in subconfluent and confluent BCE cells that had been stimulated with either 200 units/ml of TNF-α or 8μg/ml of cyclohexamide (Fig. 1). BCE cells were subconfluent at 2 days after trypsin passage but had attained their characteristic cobblestone morphology by 7 days. P-selectin mRNA was elevated 3-fold in TNF-α-stimulated cultures compared to controls at both 2 and 7 days (Panel A, compare lane 1 to 3, lane 4 to 6). The degree of upregulation varied from 2-5.4 fold in a series of four experiments. P-selectin mRNA was elevated 2-fold by prior treatment cells with cyclohexamide (Panel A, lanes 2 and 5) in both subconfluent and confluent cells (range 1-2.4 fold in four experiments). Thus, protein synthesis inhibitor cyclohexamide had only a modest effect on P-selectin mRNA compared to TNF-α.

To compare the regulation of P-selectin mRNA to that of E-selectin, the Northern blots were also probed with a [^{32}P]-labeled bovine E-selectin cDNA. As reported previously, E-selectin mRNA is upregulated by TNF-α in confluent BCE cells (11). In Figure 1 (Panel B), E-selectin mRNA was 4-fold higher in TNF-α treated cells at 2 days after trypsin passage (Panel B, lanes 1 and 3) and 28-fold higher in confluent cultures (Panel B, lanes 4 and 6). The difference in degree of induction can be attributed to the lower level of constitutive expression of E-selectin observed in confluent BCE cells (Panel B, lane 1 versus lane 4). Pre-treatment with

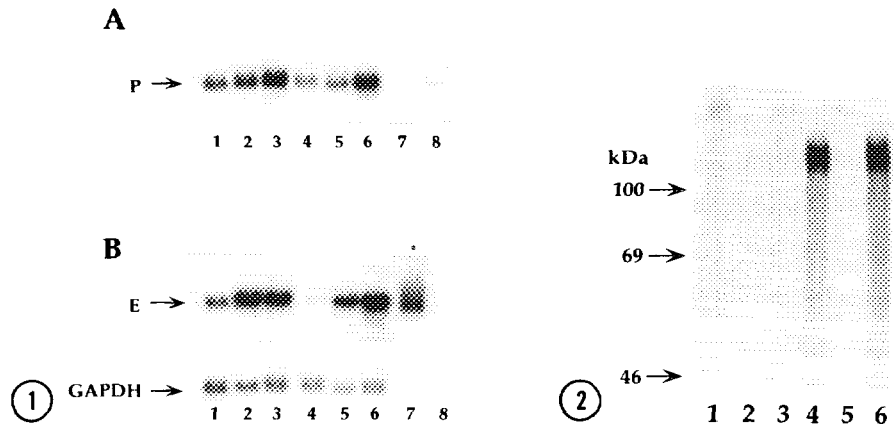

Figure 1. Northern blot analysis of P- and E-selectin mRNAs in BCE cells. BCE cells (passage 12) were split 1:6 and plated on gelatin-coated dishes in growth media. Two days (lanes 1-3) and 7 days later (lanes 4-6) cells were treated with PBS (lanes 1 and 4), 8 μg/ml cyclohexamide (lanes 2 and 5), or 200 units/ml TNF-α (lanes 3 and 6) for 5 hours. PolyA-selected RNA (3 μg) was fractionated by electrophoresis and transferred to nitrocellulose. In Panel A, the membrane was hybridized with ^{32}P -labeled bovine P-selectin cDNA. In Panel B, the blot was hybridized with ^{32}P -labeled bovine E-selectin and GAPDH cDNAs. Since bovine P- and E-selectin mRNAs are both ~3 kb (10,11), synthetic mRNA transcripts encoding bovine E-selectin (lane 7) and bovine P-selectin (lane 8) were run to assess the specificity of the hybridization conditions.

Figure 2. Bovine P-selectin polypeptide is upregulated by TNF-α and LPS. BCE cells (passage 14) were treated with PBS (lanes 1 and 2), 200 units/ml TNF-α (lanes 3 and 4), or 1μg/ml LPS (lanes 5 and 6) for 3 hours, metabolically labeled with [^{35}S]cysteine for 1 hour and then chased for 1 hour. The cells were lysed with detergent and subjected to immunoadsorption with preimmune serum (lanes 1, 3, and 5) or anti-bovine P-selectin serum (lanes 2, 4, and 6). The phosphorimager analysis of the dried SDS-PAGE gel is shown. The arrows indicate the migration of [^{14}C]methylated protein standards.

cyclohexamide resulted in an increase in E-selectin mRNA in both subconfluent (lane 2) and confluent (lane 5) BCE cells. In four experiments, the degree of induction by cyclohexamide was similar to the increase observed with TNF-α. Thus, the level of E-selectin mRNA is reduced significantly in quiescent BCE cells compared to rapidly growing cells while the level of P-selectin mRNA does not change significantly.

Ghersa and colleagues have shown that human E-selectin mRNA can be upregulated by protein synthesis inhibitors, including cyclohexamide (13). Pretreatment of human umbilical vein endothelial cells with interleukin-1β and cyclohexamide decreased the rate of E-selectin mRNA degradation and increased its rate of transcription. Their findings and the results reported here differ from an earlier study in which human E-selectin mRNA was not found to be induced by cyclohexamide (14). Cyclohexamide induction of mRNAs is thought to act by depleting the cell of short-lived regulatory proteins that function in mRNA degradation and/or

transcription. Such molecules may include RNA nucleases or repressors of transcription. The results observed in Fig. 1 indicate that steady-state levels of P-selectin mRNA are not regulated to the same degree as E-selectin by short-lived protein factors. Bovine P-selectin cDNA does not contain ATTTA sequences in its 3' untranslated region, which may protect P-selectin mRNAs from rapid degradation. Multiple AUUUA sequences in mRNAs have been found in transiently expressed molecules, including E-selectin, and are thought to confer instability. Parenthetically, the *human* P-selectin cDNA also lacks ATTTA sequences in its 3' untranslated region (15) while human and bovine E-selectin cDNAs contain 8 and 5 ATTTA sequences respectively (11,16).

The level of P-selectin polypeptide was analyzed in TNF-α and LPS-stimulated BCE cells. BCE cells were incubated in medium alone (Fig. 2 lanes 1 and 2), with 200 units/ml TNF-α (Fig. 2, lanes 3 and 4), or with 1μg/ml LPS (Fig. 2, lanes 5 and 6). The cells were then metabolically-labeled with [^{35}S]cysteine and subjected to immunoadsorption with preimmune serum (lanes 1, 3,and 5) or with anti-bovine P-selectin serum (lanes 2, 4, and 6). The specific activity of radiolabeled cell lysates was ~20% higher in stimulated cells. Equal cpms were used for each immunoadsorption. Both TNF-α and LPS resulted in a large increase in the 130 kDa bovine P-selectin.

The biosynthesis of P-selectin in control and TNF-α-stimulated BCE was analyzed by a pulse-chase metabolic-labeling experiment (Fig. 3). Confluent BCE cells were treated with PBS

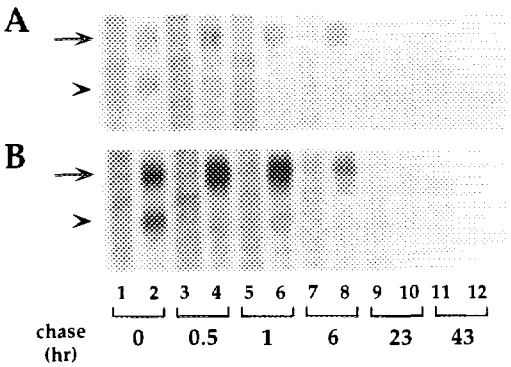

Figure 3. Biosynthesis of P-selectin in control and TNF-α-stimulated BCE cells. BCE cells (passage 12) were treated with PBS (Panel A) or 200 units/ml TNF-α (Panel B) for 3 hours, pulse-labeled for 1 hour, and then chased for the times indicated. Cells were solubilized with detergent and the lysates divided in half. One half of each time point was immunoadsorbed with preimmune serum (lanes 1, 3, 5, 7, 9, 11) and the other half was immunoadsorbed with anti-bovine P-selectin serum (lanes 2, 4, 6, 8, 10, 12). The phosphorimager analysis of the dried SDS-PAGE gel is shown. The arrow indicates the 130 kDa mature form of bovine P-selectin while the arrowhead indicates the 105 kDa precursor.

(Panel A) or with 200 units/ml of TNF-α (Panel B), pulse-labeled and then chased for 0 (lanes 1,2), 0.5 hr (lanes 3,4), 1 hr (lanes 5,6), 6 hr (lanes 7,8), 23 hr (lanes 9,10) and 43 hr (lanes 11,12). Cell lysates were immunoadsorbed with preimmune serum (lanes 1, 3, 5, 7, 9, and 11) or with anti-bovine P-selectin serum (lanes 2, 4, 6, 8, 10, 12). At each time point, the specific activity and turnover of total cellular protein ($t_{1/2}$ ~ 12 hours) did not differ between control and TNF-α activated BCE cells. Aliquots of radiolabeled cell extract containing equal cell protein were used for each immunoadsorption. In both conditions, bovine P-selectin is synthesized as a 105 kDa precursor that is processed to a 130 kDa mature form. Bovine P-selectin is smaller than human P-selectin because it contains only 6 consensus repeat domains (10). The level of P-selectin polypeptide increased dramatically after 3 hour pretreatment with TNF-α (Fig. 3 Panel B) compared to control (Fig. 3, Panel A). In TNF-α-treated cells, newly synthesized P-selectin turned over rapidly; after 6 hours of chase the level of immunoreactive P-selectin returned to near constitutive levels (compare lane 8 in Panel A to lane 8 in Panel B).

Previous studies have shown that levels of murine P-selectin mRNA and polypeptide are upregulated by TNF-α or LPS. These studies were performed on a murine endothelioma cell line transformed with the polyoma middle T antigen (6) or on whole lung tissue (3,7). We extend these findings to P-selectin in non-transformed bovine capillary endothelial cells. The regulation of P-selectin mRNA differs significantly from E-selectin mRNA expressed in BCE cells. In contrast to E-selectin, P-selectin mRNA levels are similar in growing and quiescent BCE cells. P-selectin mRNA is also less sensitive to cyclohexamide. The rapid turnover of newly synthesized P-selectin polypeptide in TNF-α-stimulated cells indicates that P-selectin function is highly regulated.

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