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Cytokine-induced β-Galactoside α-2,6-Sialyltransferase in Human Endothelial Cells Mediates α2,6-Sialylation of Adhesion Molecules and CD22 Ligands*

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Sialic acids decorating blood and cell surface proteins can play important roles in various biological processes. The inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1, as well as bacterial lipopolysaccharide, can activate vascular endothelium, increasing expression of several surface glycoproteins. Here we show that treatment of cultured human endothelial cells (HEC) with TNF-α, interleukin-1, or lipopolysaccharide causes increased expression of the enzyme β-galactoside α-2,6-sialyltransferase (α2-6STN). TNF-α was most effective, inducing a 3.5-fold enhancement of cell-associated sialyltransferase activity by 72 h. In addition, activated HEC secreted a large portion of the induced sialyltransferase activity into the medium. Analysis of labeled HEC showed both a relative and an absolute increase of α2,6-linked sialic acid on *N*-linked oligosaccharides after TNF-α stimulation. This coincided with increased expression of endothelial glycoproteins bearing *N*-linked glycans with α2,6-linked sialic acid detected by the lectin *Sambucus nigra* agglutinin. The cytokine-inducible endothelial cell adhesion molecules E-selectin, ICAM-1, and VCAM-1 are among these glycoprotein substrates for α2-6STN. These changes also correlated with a substantial increase in binding sites for CD22β, a mammalian lectin known to recognize oligosaccharides carrying multiple copies of α2,6-linked sialic acid. Northern analysis revealed increased levels of mRNA encoding α2-6STN. Thus, activation of endothelial cells during inflammatory and immunological processes may induce α2-6STN, which can participate in sialylation of other activation-dependent molecules.

Recent studies have demonstrated that specific terminal oligosaccharide sequences of glycoproteins and glycolipids participate in various adhesion events (1–4). The sialic acids are a family of closely related nine-carbon carboxylated sugars found at terminal positions of mammalian cell surface sugar chains (5). *N*-Acetylneuraminic acid (Neu5Ac)[1] is the most common sialic acid and appears to serve as a biosynthetic precursor of the other family members. Due to their negative charge, sialic acids can inhibit cell-cell interactions in a nonspecific (5, 6) or specific (7) fashion. However, sialic acids can also serve as ligands for cell-cell recognition. Several adhesion molecules bind to sugar chains with sialic acids as critical components (1–4, 8). The selectins, found on endothelium (E- and P-selectin), platelets (P-selectin), and leukocytes (L-selectin), can bind to ligands that include oligosaccharide structures such as sialyl Lewis^x (Neu5Acα2,3Galβ1,4[Fucα1,3]GlcNAcβ1-) (2–4). Also, oligosaccharides containing α2,6-linked sialic acid are critical components of ligands for CD22, an immunoglobulin (Ig) superfamily member found on a subset of B cells (8–12), and the sequence Neu5Acα2,3Galβ1,3GalNAc- serves as a primary recognition unit for the macrophage-specific adhesion molecule, sialoadhesin (13, 14).

Sialyltransferases add sialic acid to terminal positions on the oligosaccharide chains of glycoproteins and glycolipids (15). β-Galactoside α-2,6-sialyltransferase (α2-6STN) specifically catalyzes the reaction, CMP – Neu5Ac + Galβ1,4GlcNAcβ1- → CMP + Neu5Acα2,6Galβ1,4GlcNAcβ1- (16). Within the Golgi complex, α2-6STN is found as a 47-kDa type II transmembrane protein (17). Hepatocytes also release a 41-kDa soluble form of α2-6STN resulting from proteolytic cleavage of the catalytic domain from its membrane anchor (17, 18). In addition, soluble α2-6STN is one of a subset of serum glycoproteins known as acute phase reactants that are elevated during a variety of pathological processes (19–21). Acute phase reactants are generally assumed to be derived from hepatocytes (20).

Activation of vascular endothelium by cytokines and bacterial products results in an orchestrated display of new cell surface glycoproteins (22–24). In the present study, we address the effects of endothelial activation on cell surface carbohydrates and demonstrate that inflammatory cytokines act on endothelial cells to induce increased expression of cellular α2-6STN. This enhances the amount of α2,6-linked sialic acids as well as its display on newly activated adhesion molecules and on ligands of CD22β.

EXPERIMENTAL PROCEDURES

Endothelial Cell Culture and Stimulation—Primary cultures of HEC from Clonetics Corp. (San Diego, CA) were grown in dishes coated with 0.1% gelatin (Fisher, Pittsburgh, PA) using Medium 199 (Life Technologies, Inc.) with 20% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 50 μg/ml endothelial cell growth supplement, and 100 μg/ml porcine heparin (Sigma), and subcultured using trypsin/versene (Life Tech-

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; AGP, α_1-acid glycoprotein; CD22-Ig, CD22-immunoglobulin chimera; FCS, fetal calf serum; α2-6STN, β-galactoside α-2,6-sialyltransferase; HEC, cultured human endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline, TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; BSA, bovine serum albumin; HPLC, high performance liquid chromatography.

nologies, Inc.). Confluent HEC (passage 2–4) were activated by incubation at 37 °C for the indicated times with Medium 199, 20% FCS containing lipopolysaccharide (LPS; *Salmonella typhosa*, Sigma), human recombinant tumor necrosis factor-α (TNF-α) (a gift from Biogen Corp., Cambridge, MA), or human recombinant interleukin-1α (IL-1, a gift from Hoffmann La Roche, Inc.).

Sialyltransferase Assays—After stimulation of HEC in 100-mm diameter dishes (Costar Corp.), the conditioned medium (total 7.5 ml) was collected and centrifuged at 10,000 × *g* for 20 min at 4 °C, and supernatants were collected. Extraction of α2-6STN from the cells was performed according to the previous method (25). Briefly, cells were washed 4 times with ice-cold phosphate-buffered saline (PBS), scraped off in 0.5 ml of 50 mM sodium cacodylate, pH 6.5, containing 150 mM NaCl, 1% Triton X-100, and 20% glycerol, and homogenized. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C; supernatants were collected. Samples were either used immediately or stored at −80 °C for up to 2 weeks. Protein concentrations were determined using the bicinchonic acid protein assay reagent kit (Pierce Chemical Co.) with bovine serum albumin (BSA) as a standard. Sialyltransferase activity was assayed by a modification of the method of Vandamme *et al.* (25). Briefly, the standard reaction mixture contained 50 μl of cell homogenates or conditioned medium, 0.61 nmol of CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac (302.8 mCi/mmol, DuPont NEN), 6 nmol of CMP-Neu5Ac (Sigma), 1 mM 2,3-dehydro-2-deoxy-Neu5Ac (an inhibitor of sialidase, Calbiochem), 5 mM $MnCl_2$, 50 mM sodium cacodylate, 150 mM NaCl, pH 6.5, buffer, and an acceptor protein in a final volume of 100 μl. The mixture was incubated at 37 °C for 2 h with 200 μg of human asialofetuin (Sigma) as an acceptor or for 4 h using 200 μg of human asialo-$α_1$-acid glycoprotein (asialo-AGP; prepared by mild acid hydrolysis of human AGP (Sigma) (25)) as an acceptor. Under these conditions, incorporation was linear with respect to incubation time as well as to the amount of cell lysate protein or pure α2-6STN (rat liver-derived; Boehringer Mannheim). The reaction was stopped by addition of 1 ml of ice-cold 5% phosphotungstic acid in 2 N HCl. Reaction mixtures were applied to 2.4-cm Whatman filter discs (GF/C), which were then washed twice with 5% trichloroacetic acid. Filters were rinsed with water and ethanol, dried, and processed for scintillation counting. Endogenous activity (determined in the absence of exogenous acceptors, usually below 5% of activity with exogenous acceptors) was subtracted from activity in reactions including exogenous acceptors. Results are expressed as nmol of CMP-Neu5Ac transferred/mg of HEC protein/1 h. The specific activity of conditioned medium samples was calculated as the total enzyme activity in conditioned medium minus the activity of Medium 199, 20% FCS alone divided by the total HEC protein content in the particular dish.

Assessment of Sialyltransferase Activity by Lectin Precipitation and Sialidase Digestion of [^{14}C]Sialylated AGP—HEC were incubated with or without 200 units/ml TNF-α for 72 h, and the cell lysate and conditioned medium samples were prepared as described above. Sialyltransferase assays (4 h at 37 °C) were performed with 150 μl of cell homogenates (135 μg) or conditioned medium, 1.83 nmol of CMP-[^{14}C]Neu5Ac, 18 nmol of CMP-Neu5Ac, 1 mM 2,3-dehydro-2-deoxy-Neu5Ac, 5 mM $MnCl_2$, 50 mM sodium cacodylate, 150 mM NaCl, pH 6.5, buffer, and 600 μg of asialo-AGP in a final volume of 300 μl. Mixtures were then applied to a PD-10 column (Sephadex G-25M, Pharmacia LKB Biotechnology Inc.) with PBS containing 1 mg/ml BSA to remove free CMP-[^{14}C]Neu5Ac and sialidase inhibitor. The first 2 ml of effluent was discarded, and the next 1.5 ml containing [^{14}C]sialylated AGP was collected. The isolated [^{14}C]sialylated AGP (0.5 ml) was incubated with 15 μl of agarose conjugated with *Sambucus nigra* agglutinin (E. Y. Laboratories, Inc., San Mateo, CA) for 12 h at 4 °C. After five cycles of PBS washing and centrifugation, lectin-bound glycoproteins were eluted with 0.2 M lactose in PBS and the radioactivity was counted. *S. nigra* agglutinin-bound radioactivity was not eluted with PBS alone. In some cases, [^{14}C]sialylated AGP (100 μl), prepared and isolated as described above, was mixed with the same volume of 0.1 M ammonium acetate (to give a final pH of 5.4) and then digested with 20 milliunits of *Arthrobacter ureafaciens* sialidase (Boehringer Mannheim) for 12 h at 37 °C. A separate aliquot was mixed with the same volume of 0.2 M sodium cacodylate (to give a final pH of 6.9) and digested with 6 milliunits of Newcastle disease virus sialidase (prepared as per Ref. 26) for 12 h at 37 °C. The *A. ureafaciens* sialidase cleaves α2,3-, α2,6-, and α2,8-linked sialyl residues, whereas the Newcastle disease virus sialidase hydrolyzes predominantly α2,3- and α2,8-linked sialyl residues (27, 28). These specificities were validated by digesting AGP, which had been resialylated from asialo-AGP with CMP-[^{14}C]Neu5Ac in either α2,3 or α2,6 linkages by purified sialyltransferases (purified rat Galβ1,3(4)GlcNAc:α2,3-sialyltransferase was obtained from Dr. J. C. Paulson, Cytel Corp., San Diego, CA). After sialidase treatment, the radioactivity of remaining [^{14}C]sialylated AGP was measured by using a filtration procedure as described above. Under the conditions used, the Newcastle disease virus sialidase released 100% of the α2,3-linked and less than 2% of the α2,6-linked sialic acid, whereas the *A. ureafaciens* sialidase released 100% of both. These specificities were unchanged when the [^{14}C]Neu5Ac-labeled AGP standards were mixed with cell lysate (in the amounts typically used for assays) prior to the sialidase digestions.

Analysis of the Content of α2,3- and α2,6-Linked Sialic Acid in N-Linked Oligosaccharides Isolated from HEC—Confluent HEC were grown in complete medium supplemented with 16 μCi/ml [6-^{3}H]glucosamine (DuPont NEN) for 72 h with or without stimulation with 200 units/ml TNF-α. After extensively washing with PBS, the labeled cells were solubilized in lysis buffer (4% (w/v) SDS, 20 mM 2-mercaptoethanol, 10 mM HEPES pH 7.0) with heating to 80 °C for 20 min. Isolation of ^{3}H-labeled *N*-linked oligosaccharides from the glycoproteins in the lysates was performed as described (29) using the enzyme peptide:*N*-glycosidase F. The total amount of sialic acid in the released, desalted, *N*-linked oligosaccharides was determined using HPLC analysis. Briefly, sialic acids were released from isolated *N*-linked oligosaccharides with 2 M acetic acid for 3 h at 80 °C. The released sialic acids were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (Sigma) and analyzed by reverse-phase HPLC using a TSK-gel ODS-120T column (TosoHaas), as described previously (30, 31). Although this system can detect most major forms of substituted sialic acids (30, 31), the major sialic acid in isolated *N*-linked oligosaccharides from resting and TNF-stimulated HEC was Neu5Ac. Therefore, quantification of the released sialic acid was done with known standards of derivatized Neu5Ac. The ratio of α2,3 and α2,6 linkage of sialic acids on the same preparations of ^{3}H-labeled *N*-linked oligosaccharides was determined by digestions with Newcastle disease virus sialidase or *A. ureafaciens* sialidase, performed under conditions described previously (10). Sialidase-released [^{3}H]sialic acids were purified away from desialylated ^{3}H-labeled *N*-linked oligosaccharides on a Bio-Gel P-4 (Bio-Rad) column in 0.1 M ammonium formate, pH 6.5. The radioactivity of eluted [^{3}H]sialic acids was monitored. Radioactivity released by Newcastle disease virus sialidase (α2,3-linked sialic acids) or *A. ureafaciens* sialidase (α2,3- and α2,6-linked sialic acids) was subtracted from the background seen with control incubations (6% or less of the total released counts) and used to calculate the ratio of α2,3- to α2,6-linked sialic acids. Total amounts of sialic acids in each linkage were calculated using the above values and divided by total HEC protein in the starting lysate.

Lectin Blotting—HEC were washed 4 times with ice-cold PBS, incubated for 20 min at 4 °C with 0.8 ml of lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin (Sigma), 1 mM phenylmethylsulfonyl fluoride (ICN Biochemicals, Cleveland, OH), 2 μg/ml leupeptin (Sigma), and 20 mM Tris/HCl, pH 7.4), and removed by scraping. Lysates were centrifuged at 10,000 × *g* for 30 min at 4 °C to remove insoluble material and stored at −80 °C. HEC lysate (17 μg) was then diluted in Laemmli sample buffer containing 5% 2-mercaptoethanol, boiled for 3 min, and electrophoresed on a 4–20% gradient polyacrylamide gel (Daiichi Chemicals, Co., Tokyo, Japan) or 7.5% gels in the presence of SDS (SDS-PAGE) (32). After electrophoresis, the gel was soaked for 15 min in blotting buffer (100 mM Tris, 192 mM glycine and 20% (v/v) methanol). Electroblotting of proteins to an Immobilon-P membrane (Millipore Co., Bedford, MA) was performed under a constant current (1.7 mA/cm^2) in the blotting buffer for 1 h. The Immobilon-P membrane was incubated overnight at 4 °C in Tris-buffered saline (TBS; 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 20 mM Tris/HCl, pH 7.5) containing 3% BSA. The membrane was washed once with TBS, 0.1% Tween 20 and incubated for 2 h with 4 μg/ml of biotin-labeled *S. nigra* agglutinin or *Maackia amurensis* agglutinin (E. Y. Laboratories, Inc.) in TBS, 1% BSA. It was then washed three times with TBS, 0.1% Tween 20, incubated for 45 min with 1 μg/ml of streptavidin-conjugated horseradish peroxidase (Calbiochem), and washed 4 times with TBS, 0.1% Tween 20. The membrane was then incubated with chemiluminescent detection reagents (ECL Western blotting detection reagents, Amersham Corp.) according to the manufacturer's instructions and exposed to x-ray film.

Enzymatic treatment of immobilized proteins was performed as follows. After blocking with TBS, 3% BSA, the membrane was incubated with 10 milliunits/ml of sialidase (*Clostridium perfringens*, type X, Sigma) in sodium acetate buffer, pH 6.5, for 14 h at room temperature or with 10 units/ml of recombinant peptide:*N*-glycosidase F (*Flavobacterium meningosepticum*, Boehringer Mannheim) in 100 mM Tris/HCl, pH 8.5, containing 40 mM EDTA for 13 h at 37 °C. Following glycosidase treatment, the membrane was stained with biotin-labeled lectins.

Immunoprecipitation of Endothelial Cell Adhesion Molecules—HEC lysates (0.7 mg of protein; ~1 ml) were precleared using protein A-Sepharose (Pierce Chemical Co.) and incubated for 1 h at 4 °C with one of the following monoclonal antibodies (mAbs): anti-E-selectin mAb H18/7 (IgG2a; ammonium sulfate-precipitated Ig, 4 μg) (33), anti-ICAM-1 mAb E1/7 (IgG2a; purified Ig, 2 μg) (34, 35), and anti-VCAM-1 mAb E1/6 (IgG1; ammonium sulfate-precipitated Ig, 4 μg) (34, 35). Protein A-Sepharose was added, and the mixture was incubated for 1 h at 4 °C. After centrifugation at 10,000 × *g* for 3 min, the supernatant was collected. The Sepharose was washed with lysis buffer and the immunoprecipitated proteins were eluted by boiling in Laemmli sample buffer. Samples were analyzed using SDS-PAGE and stained with *S. nigra* agglutinin as described above.

Assay for CD22-Immunoglobulin Chimera (CD22-Ig) Binding to HEC—Development and characterization of a soluble CD22-Ig has been described previously (8, 10). Confluent HEC in 96-well plates were stimulated for 48 h. After washing 3 times with Hanks' balanced salt solution, cells were incubated with 5 μg/ml of CD22-Ig in Hanks' balanced salt solution, 1% BSA for 2 h at 4 °C. The cells were washed with Hanks' balanced salt solution and incubated with peroxidase-conjugated goat anti-human IgG Fc fragment-specific antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at 1/6,000 dilution. After 45 min at 4 °C, the plates were washed 3 times with Hanks' balanced salt solution followed by the addition of 0.8 mg/ml of *o*--phenylenediamine (Sigma) in 50 mM sodium citrate, 50 mM sodium phosphate buffer, pH 5.0, containing 0.015% (v/v) H_2O_2 (200 μl/well). The reaction was stopped by addition of 4 N H_2SO_4 (50 μl/well), and the plates were read at 490 nm using a V_{max} microplate reader (Molecular Devices, Inc., Menlo Park, CA). Data were expressed as $A_{490\,nm}$ value after subtracting from the background value (obtained in the presence of goat antibody alone).

Northern Analysis—Total cellular RNA was prepared from HEC grown in 150-mm diameter dishes according to the method of Chomczynski and Sacchi (36). Total RNA (10 μg) was fractionated on a 1.5% agarose gel containing formaldehyde and transferred onto nylon membranes (GeneScreen Plus®; DuPont NEN). After immobilization of the RNA by UV cross-linking, hybridization was performed overnight at 42 °C in 50% formamide, 4.8 × SSC (standard saline citrate; 15 mM sodium citrate and 150 mM NaCl), 5 × Denhardt's solution, 50 mM HEPES pH 7.3, 0.5% SDS, and 200 μg/ml denaturated herring sperm DNA. Human α2-6STN cDNA and β-actin cDNA (a gift from Dr. J. Lawler, Boston, MA) were labeled with [α-^{32}P]dCTP (DuPont NEN, 300 Ci/mmol) using a random primer labeling system. After hybridization, the membrane was washed twice with 1 × SSC containing 0.5% SDS at room temperature, rinsed twice with 0.1 × SSC containing 0.1% SDS at 50 °C, and studied by autoradiography.

RESULTS

Activation of HEC Increases Sialyltransferase Activity—Endothelial sialyltransferase activity was assessed by incorporation of label from CMP-[^{14}C]Neu5Ac into acceptor glycoproteins. TNF-α treatment resulted in a time-dependent increase in cell-associated activity directed against the exogenous acceptor asialofetuin (Fig. 1*A*). Induction of enzymatic activity was detectable by 6–12 h and continued up to 72 h (since some detachment of cells occurred beyond 72 h, we did not follow activity at later time points). The response was dose-dependent, with a half-maximal effect at 5 units/ml of TNF-α (data not shown). HEC incubated in control medium demonstrated little or no increase in cell-associated activity during this period. Abundant sialyltransferase activity was found in the conditioned medium of the HEC by 48–72 h and was much increased in media from TNF-stimulated monolayers (Fig. 1*B*). As shown in Table I, treatment of HEC with TNF-α for 72 h resulted in a 3.5-fold increase in cell-associated sialyltransferase activity when assessed either with asialofetuin, which contains three triantennary complex-type *N*-linked glycans and three *O*-linked glycans (37), or with asialo-AGP, which contains only *N*-linked glycans (38). IL-1 as well as bacterial endotoxin (LPS) also stimulated increases in activity in cells (Table I) and in conditioned medium (data not shown) using both acceptors.

Induced HEC Sialyltransferase Adds α2,6-Linked Sialic Acid to Acceptor Glycoproteins—The majority of cell surface glycoproteins contain sialic acids linked α2,3 or α2,6 to Gal or GalNAc. Certain cells also contain extended chains of α2,8-linked sialic acid (polysialic acid) (7). These different linkages are generated by distinct sialyltransferases. The linkage specificity of the induced endothelial sialyltransferase activity was examined using specific lectins and sialidases. The lectin *S. nigra* agglutinin recognizes α2,6-linked sialic acid but not α2,3-linked sialic acid (39). Incorporation of [^{14}C]-sialic acid into asialo-AGP by TNF-induced (72 h) HEC sialyltransferase increased *S. nigra* agglutinin-precipitable counts (cell lysates, 4-fold; conditioned medium, 2.5-fold) compared with the sialyltransferase activity of resting HEC. The [^{14}C]sialylated AGP was treated with sialidases from *A. ureafaciens*, which hydrolyzes α2,3-, α2,6-, and α2,8-linked sialic acid, or with Newcastle disease virus sialidase, which cleaves α2,3- and α2,8-linkages (28). As shown in Fig. 2, *A. ureafaciens* sialidase completely removed the sialic acids from the [^{14}C]sialylated AGP products, whereas Newcastle disease virus sialidase had little or no effect. Thus, the measured endothelium-derived sialyltransferase activity adds predominantly α2,6-linked sialic acid to the acceptor.

Effect of TNF-α on the Content of α2,3- and α2,6-Linked Sialic Acid in N-Linked Oligosaccharides of Glycoproteins—*N*-Linked oligosaccharides were released by peptide:*N*-glycosidase F from total glycoproteins of resting or TNF-stimulated HEC labeled with [^{3}H]glucosamine for 72 h. The total amount and ratio of α2,3- to α2,6-linked sialic acids in the released oligosaccharides were examined as described under "Experimental Procedures." As summarized in Table II, the *N*-linked oligosaccharides carried a surprisingly large fraction of their

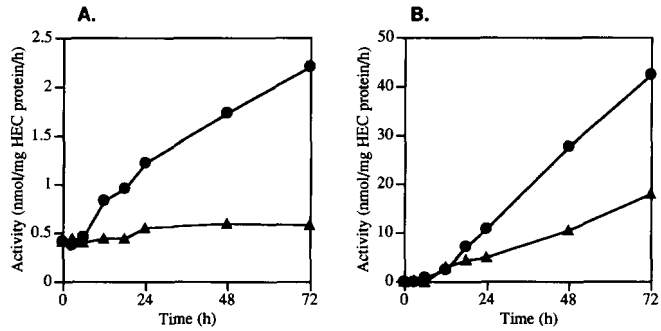

FIG. 1. **Effect of TNF-α on sialyltransferase activity in HEC.** HEC were incubated with (●) or without (▲) 200 units/ml TNF-α for the indicated times. The conditioned medium was collected, the cells were washed with ice-cold PBS and homogenized, and sialyltransferase activity in the cell lysates (*A*) and the conditioned medium (*B*) was assayed by incorporation of CMP-[^{14}C]Neu5Ac into asialofetuin (see "Experimental Procedures" for details). Results are expressed as nmol of CMP-Neu5Ac transferred per mg of HEC protein/h. Data are the mean values from a representative experiment performed in duplicate. Similar results were obtained in two additional experiments.

TABLE I
Effects of various stimuli on cellular sialyltransferase activity in HEC

HEC were incubated with M199, 20% FCS alone or the same medium containing 200 units/ml TNF-α, 1.7 ng/ml IL-1, or 3 μg/ml LPS for 72 h. Sialyltransferase activity in cell lysates were assayed by incorporation of label from CMP-[^{14}C]Neu5Ac into asialofetuin or asialo-AGP. Data are the mean ± S.E. from a representative experiment performed in triplicate. At least two experiments were performed for each stimulant at this time period.

Stimulus	Acceptor	
	Asialofetuin	Asialo-AGP
	nmol Neu5Ac/mg HEC protein/h	
None	0.63 ± 0.01	1.11 ± 0.05
TNF-α	2.19 ± 0.04	3.87 ± 0.10
IL-1	1.68 ± 0.04	2.88 ± 0.03
LPS	1.51 ± 0.01	2.49 ± 0.01

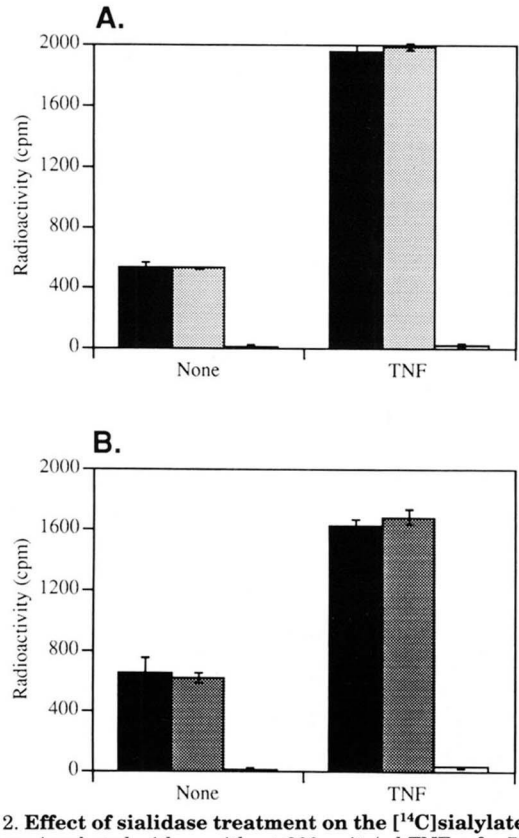

FIG. 2. **Effect of sialidase treatment on the [^{14}C]sialylated AGP.** HEC were incubated with or without 200 units/ml TNF-α for 72 h, and cell lysate (*A*) and conditioned medium samples (*B*) were prepared. [^{14}C]Sialylated AGP was prepared from asialo-AGP using these samples and isolated on PD-10 columns. Aliquots (100 μl) of the [^{14}C]sialylated AGP were treated with buffer alone (*black columns*), Newcastle disease virus sialidase (*hatched columns*) or *A. ureafaciens* sialidase (*open columns*), as described under "Experimental Procedures." The radioactivity remaining with [^{14}C]sialylated AGP was then measured. Data are the mean ± S.D. from a representative measurement performed in triplicate. Similar results were obtained in two additional experiments.

TABLE II

Effect of TNF-α stimulation on the content of α2,3- and α2,6-linked sialic acid in N-linked oligosaccharides of total HEC glycoproteins

N-Linked oligosaccharides were released from total glycoproteins of resting or TNF-stimulated HEC. ([^{3}H]Glucosamine labeling was used as a tracer to follow the purification.) Total sialic acid contents of the released oligosaccharides and the ratio of α2,3-/α2,6-linked sialic acid were determined as described under "Experimental Procedures." Data are the mean ± S.D. of three separate experiments. The asterisk indicates a statistically significant ($p = 0.01$, Student's t test) increase in α2,6-linked sialic acid content in TNF-α treated HEC.

Stimulus	Sialic acids[a]	
	α2,3-Linked	α2,6-Linked
	pmol Neu5Ac/μg HEC protein	
None	0.71 ± 0.04	0.76 ± 0.08
TNF-α	0.75 ± 0.06	1.07 ± 0.09*

[a] In *N*-linked oligosaccharides.

sialic acids in α2,3 linkage (endosialidase treatments ruled out the presence of extended α2,8-linked polysialic acids, data not shown). However, TNF-α activation induced a significant increase only in the relative and absolute amount of α2,6-linked sialic acid (Table II).

Activation of HEC Increases Expression of Specific Glycoproteins with α2,6-Linked Sialic Acid—HEC lysates, prepared from unstimulated and TNF-stimulated (24 h) monolayers, were resolved by SDS-PAGE, blotted onto membranes, and stained with two lectins: *S. nigra* agglutinin, which recognizes α2,6-linked sialic acid (39), and *M. amurensis* agglutinin, which

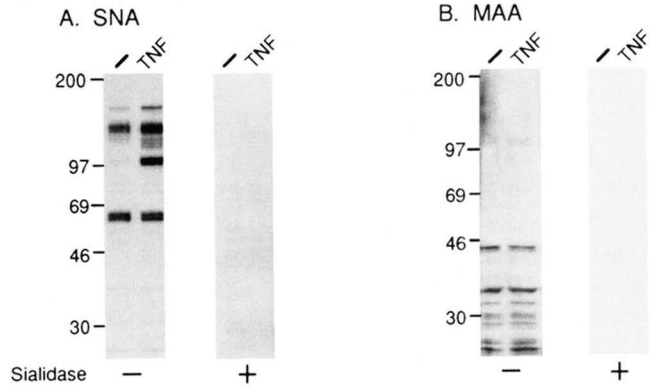

FIG. 3. ***S. nigra* agglutinin and *M. amurensis* agglutinin lectin blots of endothelial glycoproteins.** HEC were incubated with or without 200 units/ml TNF-α for 24 h. Cell lysate proteins were resolved by SDS-PAGE (4–20% polyacrylamide gradient) and blotted on membranes. After treatment with (+) or without (–) sialidase, the membranes were stained with *S. nigra* agglutinin (*A*) or *M. amurensis* agglutinin (*B*). Molecular weight markers are: myosin (200 kDa), phosphorylase *b* (97 kDa), BSA (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

recognizes sialic acid in α2,3 linkage to Galβ1,4GlcNAc (40). As shown in Fig. 3*A*, *S. nigra* agglutinin staining of endothelial glycoproteins was considerably enhanced by TNF-mediated activation. Notably, several glycoproteins of estimated molecular weight between 100 and 120 kDa stained prominently on blots prepared from activated endothelial cells but were barely detected on those prepared from control endothelial cells. At least two other glycoproteins (approximately 140 and 170 kDa) showed substantial staining in control preparations, which were enhanced in activated endothelial cells. A prominent band(s) at approximately 60 kDa showed little or no increase in *S. nigra* agglutinin staining after endothelial activation. Similar patterns of *S. nigra* agglutinin staining were observed after activation by IL-1 or LPS (data not shown). By contrast, TNF-α activation resulted in little or no effect on *M. amurensis* agglutinin staining, which predominantly recognized lower molecular weight glycoproteins (Fig. 3*B*). *S. nigra* agglutinin and *M. amurensis* agglutinin staining was abolished after sialidase treatment of immobilized glycoproteins (Fig. 3), consistent with a requirement for terminal sialic acids. In addition, treatment with peptide:*N*-glycosidase F abrogated *S. nigra* agglutinin staining of most glycoprotein species but had only a modest effect on staining of the 60-kDa constitutively expressed band(s) (data not shown). *S. nigra* agglutinin staining was also blocked by co-incubation with 1 mM Neu5Acα2,6Galβ1,4Glc-$(CH_2)_8COOCH_3$ but not Neu5Acα2,3Galβ1,4GlcNAc-$(CH_2)_8COOCH_3$ (gifts from Chembiomed, LTD, Alberta, Canada). As shown in Fig. 4, TNF-induced expression of glycoproteins bearing α2,6-linked sialic acid was time-dependent, with certain *S. nigra* agglutinin-reactive species increasing throughout a 72-h treatment period.

Induced Endothelial Cell Adhesion Molecules Show Incorporation of α2,6-Linked Sialic Acid—The cytokine-induced expression and apparent molecular weights (100–120 kDa) of certain *S. nigra* agglutinin-reactive glycoproteins suggested their identities. The endothelial adhesion molecules E-selectin (41), ICAM-1 (42), and VCAM-1 (34, 35, 43) were immunoprecipitated from HEC with specific mAbs and stained with *S. nigra* agglutinin after SDS-PAGE and blotting. As shown in Fig. 5*A*, each of these adhesion molecules could be stained with *S. nigra* agglutinin. In agreement with its known kinetics of expression (33), E-selectin and its associated *S. nigra* agglutinin reactivity were found in greater abundance in endothelial preparations that had been stimulated with TNF-α for 4 h

(data not shown) than in those that had been stimulated for 24 h (Fig. 5*A*). ICAM-1 immunoprecipitated from TNF-stimulated endothelium was strongly stained by *S. nigra* agglutinin (Fig. 5*A*). As shown in Fig. 5*B*, adsorption of endothelial lysates with anti-ICAM-1 mAb resulted in diminished *S. nigra* agglutinin staining of the 100-kDa protein(s), but not of other *S. nigra* agglutinin binding proteins.

Activation of HEC Increases CD22-Ig Binding—Recent studies identified CD22β as a mammalian lectin that recognizes specific *N*-linked oligosaccharides containing α2,6-linked sialic acid (10, 12). As shown in Fig. 6, treatment with TNF-α, IL-1, and LPS resulted in an enhancement in binding of a CD22-Ig chimera (CD22-Ig) to HEC. This CD22-Ig binding was dependent on the CD22β region, since other fusion proteins (CD8-Ig and E-selectin-Ig) could not bind to resting or TNF-stimulated HEC (data not shown). Pretreatment with 0.1 units/ml *Vibrio cholerae* sialidase (30 min at 37 °C) or mild periodate oxidation (2 mM in PBS, 30 min at 4 °C) completely abolished CD22-Ig binding to both resting and TNF-stimulated HEC (data not shown), confirming the role of sialic acids and their side chains in the binding (10, 12). In addition, CD22-Ig binding was blocked by co-incubation with 1 mM Neu5Acα2,6Galβ1,4Glc-$(CH_2)_8COOCH_3$ (87 ± 4% inhibition) but not Neu5Acα2,-3Galβ1,4GlcNAc-$(CH_2)_8COOCH_3$, indicating that CD22-Ig recognizes the ligands containing α2,6-linked sialic acid.

Effect of TNF-α on the α2-6STN mRNA Levels in HEC—Northern blot analysis of total RNA from HEC (Fig. 7*A*) showed that TNF-α treatment caused a time-dependent increase in expression of α2-6STN mRNA. Increased mRNA was detected by 6 h, slightly preceding the increase in cellular sialyltransferase activity (Fig. 1*A*). The induction of mRNA continued up to 72 h. IL-1 and LPS also enhanced the expression of α2-6STN mRNA (Fig. 7*B*). The level of α2-6STN mRNA in unstimulated HEC was not altered during a 72-h incubation (data not shown). Thus, the increase of α2-6STN activity is associated with increase of the corresponding mRNA.

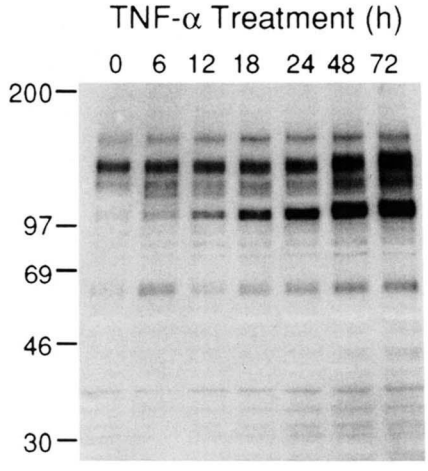

FIG. 4. ***S. nigra* agglutinin lectin blots of glycoproteins from HEC stimulated with TNF-α for various time periods.** HEC were incubated with 200 units/ml TNF-α for the indicated times. The cell lysate proteins were resolved by SDS-PAGE (4–20% polyacrylamide gradient) and analyzed by blotting with *S. nigra* agglutinin.

DISCUSSION

Endothelial cells undergo profound changes in response to cytokines like IL-1 and TNF (22–24). These changes allow the endothelium to help orchestrate the movement of fluid and leukocytes that characterizes inflammatory and immunological reactions. Here we have demonstrated that cytokines also regulate endothelial glycosylation. Specifically, TNF-α and IL-1, as well as LPS, were shown to increase α2-6STN mRNA and sialyltransferase activity. The TNF-α-induced sialyltransferase activity was detected using as acceptor proteins asialofetuin, which contains both *N*- and *O*-linked glycans (37), and asialo-AGP, which contains only *N*-linked glycans (38). Treatments with linkage-specific sialidases and binding to the lectin *S. nigra* agglutinin demonstrated that most, if not all, of the sialic acid incorporated into asialo-AGP by sialyltransferase was in α2,6-linkage. Induction of endothelial α2,6-sialyltransferase activity was found to coincide with the following observations. First, TNF-α stimulation resulted in an enhancement of α2,6-linked sialic acid content in *N*-linked oligosaccharides isolated from glycoproteins (Table II). Second, TNF-α induced an increase in the *S. nigra* agglutinin lectin staining of certain endothelial glycoproteins (Fig. 3*A*). In most of these proteins, the binding of *S. nigra* agglutinin was susceptible to peptide:*N*-glycosidase F, indicating that the α2,6-linked sialic acids were on *N*-linked oligosaccharides. In contrast, the binding activity of *M. amurensis* agglutinin lectin (α2,3-linked sialic acid-specific) was not altered by TNF-α stimulation (Fig. 3*B*). Third,

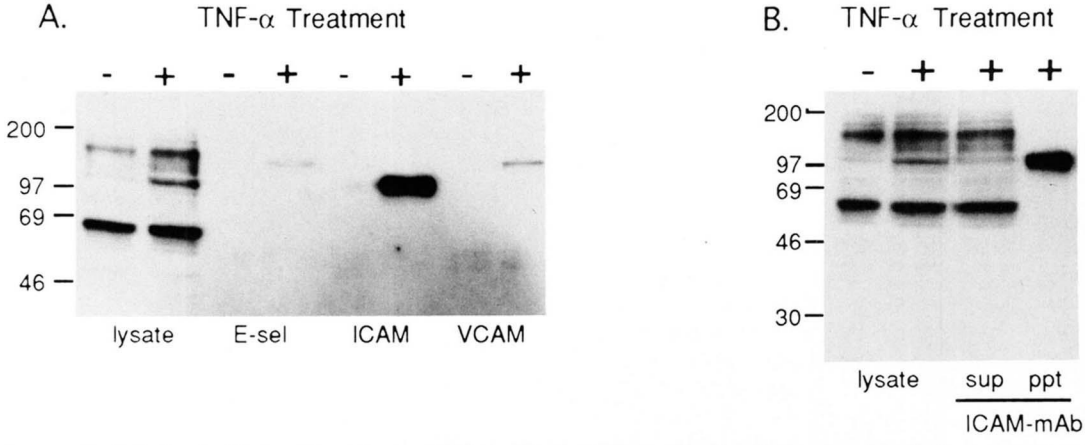

FIG. 5. ***S. nigra* agglutinin lectin blots of immunoprecipitated endothelial cell adhesion molecules.** *A*, *S. nigra* agglutinin staining of total HEC lysate, E-selectin, ICAM-1, and VCAM-1. Endothelial cells were treated for 24 h with (+) or without (−) 200 units/ml TNF-α, and the adhesion molecules were immunoprecipitated with specific mAb. Each preparation was resolved by SDS-PAGE (7.5% polyacrylamide) and analyzed by blotting with *S. nigra* agglutinin. *B*, specific inhibition of *S. nigra* agglutinin staining of the 100-kDa glycoprotein by adsorption with anti-ICAM-1 mAb. After treatment of HEC with TNF-α for 24 h, ICAM-1 was depleted from the lysates by immunoprecipitation with anti-ICAM-1 mAb. Protein A-Sepharose with bound antigen antibody was centrifuged, and samples of the depleted supernatant (*sup*) and the immunoprecipitated ICAM-1 (*ppt*) were resolved by SDS-PAGE (7.5% polyacrylamide) and analyzed by blotting with *S. nigra* agglutinin. The left two lanes are *S. nigra* agglutinin blots of the lysates from unstimulated and TNF-α-stimulated HEC.

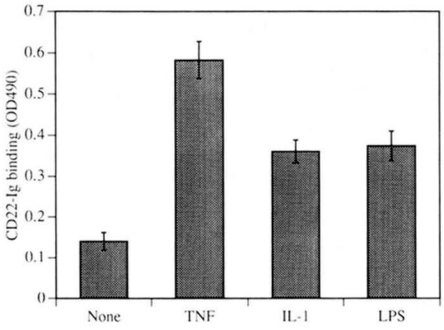

FIG. 6. **Effects of various stimuli on CD22-Ig binding to HEC.** HEC were incubated with M199, 20% FCS alone or the same medium containing 200 units/ml TNF-α, 1.7 ng/ml IL-1 or 3 μg/ml LPS for 48 h. CD22-Ig binding was assayed as described under "Experimental Procedures." Data are the mean ± S.E. from a representative experiment performed in triplicate. At least two experiments were performed for each stimulant.

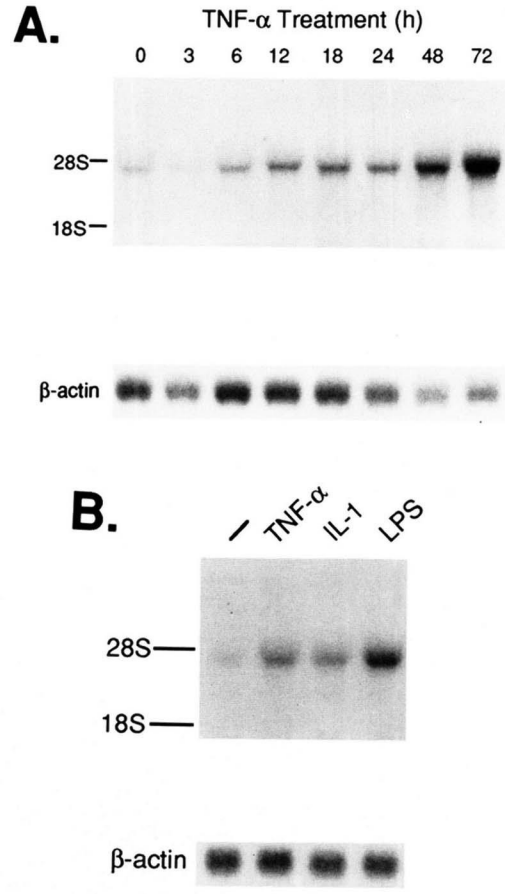

FIG. 7. **Effects of cytokines and LPS on α2-6STN mRNA levels.** *A*, HEC were incubated with 200 units/ml TNF-α for the indicated times. Total RNA (10 μg/lane) was analyzed by Northern blotting using α2-6STN cDNA or β-actin cDNA as a probe. Quantification by densitometry of autoradiogram of blots (with normalization to β-actin mRNA content) showed that α2-6STN message was enhanced 7.2-fold after a 72-h stimulation with TNF-α. *B*, HEC were incubated with M199, 20% FCS alone or the same medium containing 200 units/ml TNF-α, 1.7 ng/ml IL-1, or 3 μg/ml LPS for 24 h.

TNF-α activation resulted in increased binding of CD22-Ig, which recognizes specific *N*-linked oligosaccharide structures containing α2,6-linked sialic acid (10, 12). Taken together, these data indicate that cytokine activation of vascular endothelial cells increases the expression of α2-6STN, which participates in glycosylation of several glycoproteins. The expression patterns of other glycosyltransferases in activated vascular endothelium deserves attention in the future.

Although α2-6STN is synthesized as a transmembrane molecule and probably functions in this form within the Golgi apparatus (44), a 41-kDa active form is released by the action of a cathepsin D-like proteinase (21). Recent studies have identified this soluble α2-6STN as one of the acute-phase reactants (19). The elevation of α2-6STN in the blood of patients with various acute inflammatory conditions appears to depend on its inducible expression and active release from cells (19, 45, 46). Previous studies have shown that hepatocytes respond to glucocorticoids and hepatocyte-stimulating factor with a marked increase in production of a group of glycoproteins that includes α2-6STN (47, 48). In the present study, treatment of HEC with inflammatory mediators caused a substantial increase in α2-6STN enzyme activity in the conditioned medium. In fact, the amounts found in the conditioned medium at 72 h of stimulation were about 20 times that remaining in the cells (Fig. 1). Thus, it is likely that activated vascular endothelial cells contribute to the increased serum α2-6STN levels in acute phase reactions. In addition, normal serum levels of this enzyme may be explained, in part, by the constitutive synthesis and release of α2-6STN from endothelial cells.

Lectin blotting experiments revealed the presence of α2,6-linked sialic acid both on constitutively expressed proteins and on the cytokine-inducible adhesion molecules E-selectin, ICAM-1, and VCAM-1. Unactivated endothelial cells express little or no E-selectin. With activation, E-selectin expression is abundant by 4–6 h and declines toward basal levels by 24–48 h (33). Modest amounts of ICAM-1 and VCAM-1 are typically found on unstimulated cultured endothelial cells, and exposure to activating cytokines causes dramatic increases, which are sustained for several days (24). The kinetics of expression of endothelial sialyltransferase activity, as well as of the mRNA encoding α2-6STN, are similar to those of ICAM-1 and VCAM-1. Unstimulated cells were found to contain low levels of mRNA for α2-6STN and low levels of sialyltransferase activity. TNF-α treatment resulted in large increases that were first observed after 6–12 h and continued through 72 h. Thus, cytokine-activated endothelial cells coordinately express adhesion molecules and α2-6STN that contributes to their post-translational modification. The constitutively expressed α2-6STN is likely to be responsible for sialylation of induced molecules during the early hours of activation and, therefore, may be particularly important for E-selectin. It is also likely that the induced α2-6STN contributes to the sialylation of other induced or noninduced glycoproteins (140- and 170-kDa proteins in Fig. 3*A*) or of glycolipids in the activated endothelial cells. Considerable changes have been reported in the expression of glycoproteins in migrating endothelial cells (49) and of glycosphingolipids in interferon-γ-treated HEC (50).

In human B lymphocytes, sialyltransferase activity is upregulated, and soluble enzyme is released into the medium during activation (51). Expression of the α2-6STN in B cells has been reported to regulate the generation of multiple cell surface differentiation antigens, such as CDw75 and CD76 (52, 53). The induced expression of α2-6STN on activated endothelium may also have functional consequences. First, the present study demonstrated an increase of CD22-Ig binding to cytokine-activated endothelial cells in an α2,6-linked sialic acid-dependent manner (Fig. 6), suggesting that cytokine-induced α2-6STN contributes to generation of ligand(s) for CD22. Since CD22 is expressed on resting mature B cells and is thought to play a role in cell-cell interactions and B cell activation (54), it is tempting to speculate that $CD22^+$B cells may bind to cytokine-activated vascular endothelium. In this regard, our recent work indicates that the number and distribution of α2,6-linked sialic acids can dramatically alter the recognition of oligosaccharides

by CD22 (12). This may also explain why a relatively small increase (1.4-fold) in the amount of α2,6-linked sialic acids after TNF stimulation (Table II) causes such a marked increase (>4-fold) in CD22-Ig binding. Also, it has been suggested that L-selectin (expressed on leukocytes (3)) can recognize a cytokine-inducible and sialidase-sensitive ligand on endothelial cells (55). The possibility that α2-6STN contributes to the generation of ligand(s) for L-selectin deserves attention. Finally, increasing evidence suggests that oligosaccharides modify the expression and function of a variety of cell surface and soluble proteins (56). Ultimately, studies of activation-induced changes in endothelial glycosylation must be interpreted in the context of the multifaceted changes that occur at vessel wall during inflammatory and immunological processes.

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