High endothelial cells synthesize and degrade sLex. Putative implications for L-selectin-dependent recognition

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Abstract L-selectin guides lymphocytes into peripheral lymphoid tissues by recognizing glycoprotein ligands decorated with 6-sulfated sialyl Lewis x (sulfo sLex). Here we have used a rat peripheral lymph node high endothelial cell line (Ax) to study in detail the synthesis, expression and degradation of sLex epitope. We show here that Ax cells possess active α(1,3)fucosyltransferase Fuc-TVII, the enzyme responsible for the final fucosylation of sialyl-*N*-acetyllactosamine during sLex synthesis, and express sLex on the cell surface. Furthermore, these cells degrade sLex, primarily by desialylating it to neutral Lex epitopes by α(2,3)sialidase(s).

Key words: Endothelium; Inflammation; α(1,3)fucosyltransferase; α(2,3)sialidase; Sialyl Lewis x

1. Introduction

Leukocyte tethering to and rolling on endothelial cells, mediated by selectins and their oligosaccharide-bearing counterreceptors, initiates the extravasation cascade. L-selectin, which is expressed on leukocytes, recognizes endothelial mucins GlyCAM-1, CD34 and MAdCAM-1 and guides lymphocytes to lymphoid tissues via high endothelial cells [1,2]. The oligosaccharides on these endothelial mucins contain α(2,3)sialic acid, α(1,3)fucose and 6-sulfate, which are crucial in the primary recognition [3]. The specialized endothelial cells in lymph nodes express sialyl Lewis x (sLex) epitope [4], which is one of the simplest oligosaccharides that interact with L-selectin. At parenchymal tissues, inflammatory stimuli can de novo induce endothelium to express sLex-containing glycans [5,6]. The final reaction in the synthesis of sLex is the α(1,3)fucosylation of a sialyl-*N*-acetyllactosamine acceptor. Of the five cloned human α(1,3)fucosyltransferases (Fuc-TIII–Fuc-TVII), Fuc-TVII has been suggested to have a pivotal role in the biosynthesis of endothelial ligands of L-selectin [7].

A high endothelial cell line Ax, established from the rat peripheral lymph nodes [8], has been shown to incorporate sulfate and bind L-selectin-IgG fusion protein, among other features typical of high endothelial cells [9]. In this study, Ax cells were also shown to express sLex-containing glycans. These cells possess Fuc-TVII and synthesize sLex from sialyl-*N*-acetyllactosamine acceptor. In addition, Ax cells degrade their own sLex epitopes, primarily by desialylating them to Lex, while a small amount also undergoes a defucosylation. The enzymatic machinery to control the expression of sLex motifs on various cell surface scaffolds might be important in the regulation of selectin-dependent leukocyte extravasation.

2. Materials and methods

2.1. Cell cultures

The Ax cell line was generated and cultured as previously described [9]. Fuc-TIV and Fuc-TVII transfected CHO cells were kindly provided by Dr. J.B. Lowe, Howard Hughes Medical Institute, Ann Arbor, MI,, USA [10,11].

2.2. Antibodies and flow cytometry

mAb 2H5 against sLex was a gift from Dr. R. Kannagi, Research Institute, Aichi Cancer Center, Nagoya, Japan, mAb FH-6 against di-sLex (NeuNAcα2,3Galβ1,4(Fucα1,3) GlcNAcβ1,3Galβ1,4-(Fucα1,3)GlcNAc) was kindly provided by Dr. S. Hakomori, The Biomembrane Institute, Seattle, WA, USA, and mAb CSLEX1 against sLex/di-sLex was a gift from Dr. Olli Lassila, University of Turku, Finland. mAb KM-93 against sLex/di-sLex was provided by Dr. K. Shitara, Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan. Anti-murine Fuc-TVII antibody was kindly provided by Dr. J.B. Lowe [12]. Flow cytometry was performed as previously described [13]. Negative controls included isotype matched sialyl Lewis a monoclonal antibody or deletion of the relevant primary antibody.

2.3. Reverse transcriptase PCR

Total RNA from Ax cells was extracted and cDNA was synthesized according to standard protocols. Two μl of the resulting cDNA was subjected to 30 cycles of PCR amplification. The sequence of the forward primer for Fuc-TVII was GTGCATGTGG ATGACTTTGG (no. 948–967 in U08112 in GenBank) and the reverse primer for Fuc-TVII TGAAACCAAC CCTCAAGGTC (no. 1156–1137 in U08112). The Fuc-TVII cDNA was used as a positive control and PCR performed on the cDNA prepared without reverse transcriptase as a negative control. Each PCR cycle consisted of a denaturing step at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min. The sequence of the forward primer for GAPDH was GTCTTCACCACC ATGGAGAAGGCT (no. 365–388 in X02231) and for the reverse primer (TGTAGCCCAGGA TGCCCTTTAGTG (no. 893–870 in X02231). Each cycle for GAPHD synthesis consisted of 1 min at 92°C, 1 min at 55°C and 1 min at 72°C. The amplified products were electrophoresed on 1.5% agarose gel stained with ethidium bromide and visualized under a UV-lamp. The cDNAs for human Fuc-TIII–Fuc-TVII were provided by Dr. J.B. Lowe.

2.4. Northern blot

Total RNA was extracted according to standard protocols. 15–30 μg samples of RNA were size-fractionated on 0.8% agarose-formaldehyde gels, transferred on a nylon filter, dried and baked at +80°C

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Abbreviations: Fuc, fucose; Fuc-T, fucosyltransferase; Gal, galactose; GlcNAc, *N*-acetylglucosamine; HEV, high endothelial venule; Lex, Lewis x; NeuNAc, *N*-acetylneuraminic acid; sLex, sialyl Lewis x

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and hybridized with cDNA probe labelled with [α-^{32}P]CTP. After washings the filter was exposed to X-ray films.

2.5. α(1,3)fucosyltransferase assay

Cell extracts for fucosyltransferase assays were prepared by lysing 10^7 Ax cells in 300 μl of 1% Triton X-100 with 0.1 mM PMSF. Acceptor NeuNAcα2-3Galβ1-4GlcNAc from Oxford Glycosystems (250 nmol/reaction), donor GDP-[^{14}C]fucose from NEN, Boston, MA, USA (specific activity 283 mCi/mmol, 0.35 nmol/reaction) and cell lysate were incubated 5 h at 37°C. The reaction and characterization of the reaction products were performed as described earlier [13].

2.6. Synthesis of the radiolabelled sLex heptasaccharide

The primer hexasaccharide NeuNAcα2-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc was synthesized enzymatically from *N*-acetyllactosamine with human serum β(1,3)*N*-acetylglucosaminyltransferase, bovine milk β(1,4)galactosyltransferase purchased from Sigma, rat serum β(1,6)*N*-acetylglucosaminyltransferase, and human placenta α(2,3)sialyltransferase [14]. The hexasaccharide was α(1,3)fucosylated with partially purified α(1,3/4)fucosyltransferase from human milk. The acceptor saccharide (1.2 nmol), donor GDP-[^{14}C]fucose (2.4 nmol), $MnCl_2$ (16 mM), MOPS-NaOH (50 mM, pH 7.5), NaCl (0.1 M), BSA (0.5 mg/ml), NaN_3 (0.04%) and 15 μU of enzyme in 10 μl of total volume were incubated for 72 h at 37°C. The reaction mixture was diluted with H_2O and the radiolabelled sLex glycan was purified with an ion-exchange column.

2.7. Glycosidase assay with the radiolabelled sLex heptasaccharide

Nine pmol/reaction of radioactive sLex-bearing heptasaccharide substrate (NeuNAcα2-3Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc) was dried in the reaction tube and 10 μl of 100 mM sodium acetate buffer (pH 5.0) and 10 μl of Ax cell lysate (see above) were added. As a negative control, the substrate was incubated with the buffer and detergent without the cells. The reactions were incubated for 18 h at 37°C. Termination of the reaction and characterization of the reaction products were performed as described previously [13].

3. Results and discussion

3.1. Ax cells express sLex motifs

High endothelial cells synthesize mucin-like glycoproteins, such as GlyCAM-1 and CD34, which act as ligands for L-selectin. Post-translational decorations on these glycoproteins, containing sLex or sulfated sLex, are of vital importance in regard to selectin-dependent cell recognition [1,2]. By using several anti-sLex antibodies we demonstrated that Ax cells expressed sLex/di-sLex epitope recognized by mAbs KM-93, FH-6 and 2H5. On the other hand, one anti-sLex mAb, CSLEX1, showed no reactivity (Fig. 1).

Table 1
Comparison of the murine and proposed rat Fuc-TVII DNA sequence; residues that exactly match are marked with a line; in the overall 202 residues 76% homology was seen between the murine and rat sequences

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Mouse FTVII  2876 gacgacttcagctctgcccg..tgaactggctgtcttcctcgtcagcatg 2923
Rat   FTVII     :|||  | ::| ||:  |||   || ||||| |  ||||||    ||||| 
                1 NACGCGTGNNGGTCNCACCGGCAGAGCTGGCGGCTTTCCTCACTGGCATG 50

Mouse FTVII  2924 aatgagagtcgttatcgtggcttctttgcttggcgagaccggctccgtgt 2973
Rat   FTVII       |||||||| || || |  |||||||||| ||||| ||| |||||||| ||
                 51 AATGAGAGCCGATACCAACGCTTCTTTGCCTGGCGTGACAGGCTCCGCGT 100

Mouse FTVII  2974 gcggctcctgggtgactggagggagcgcttctgcaccatctgtgcccgct 3023
Rat   FTVII       ||| ||  |   |||||| |||| || |||||  |||||||||| |||||
                101 GCGACTGTTCACCGACTGGCGGGAACGTTTCTGTGCCATCTGTGACCGCT 150

Mouse FTVII  3024 acccttacttgccccgcagccaggtctatgaagaccttgaaagctggttc 3073
Rat   FTVII       ||||  || | |||||||||||| |||||||| ||||||||  | |||||
                151 ACCCACACCTACCCCGCAGCCAAGTCTATGAGGACCTTGAGGGTTGGTTT 200

Mouse FTVII  3074 ca 3075
Rat   FTVII       ||
                201 CA 202
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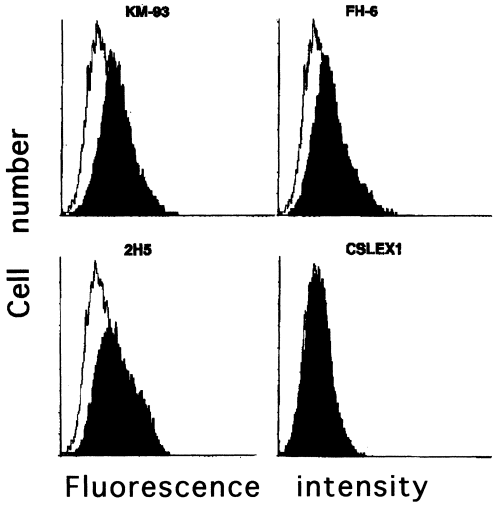

Fig. 1. Expression of sLex on the surface of Ax cells analyzed by flow cytometry. For negative control the cells were incubated with the isotype matched control antibody (white curve). One representative experiment out of three is presented.

3.2. Ax cells express Fuc-TVII transcripts

Studies in gene knock-out mice suggest an essential role of Fuc-TVII in L-selectin ligand biosynthesis [7]. Both in situ hybridization and immunohistochemistry have suggested the presence of Fuc-TVII in the high endothelial venules in murine lymph nodes [12]. To analyze whether Fuc-TVII is present in the cultured rat high endothelial cells, we isolated mRNA from these cells, transcribed it to cDNA, and subjected it and the control human Fuc-TVII cDNA to amplification by Fuc-TVII primers. One cDNA band of the expected size from Ax cDNA as well as from the control Fuc-TVII cDNA was synthesized, while nothing was synthesized in the corresponding negative control (Fig. 2a).

As the cloned α(1,3)fucosyltransferases are highly homologous, we size-fractionated the PCR products and showed with Southern blotting, that PCR products hybridized only with the full length human Fuc-TVII, but not with Fuc-TIII, Fuc-TIV, Fuc-TV or Fuc-TVI cDNA probes (Fig. 2b). The alignment of our new sequence from the PCR amplification and murine Fuc-TVII DNA show 76% homology suggesting that the former represented the previously unidentified rat Fuc-TVII sequence (Table 1).

Reverse transcriptase PCR experiments show that though transcripts corresponding to all five cloned α(1,3)fucosyltransferases can be identified from leukocytes, only two of them are present in quantities large enough to be detected by Northern blot analyses [11,15]. To analyze the actual level of Fuc-TVII transcript expression in cultured high endothelial cells we performed Northern blots, and as shown in Fig. 2c, two strong bands at 1.5 and 3.0 kb were detectable, as previously shown in HL60 cells as well [11]. The expression of Fuc-TVII has been studied also in other endothelial cells. Cytokine activation of human umbilical vein endothelial cells or transfection of Fuc-TVII cDNA to sLex-negative endothelial cell line induce Fuc-TVII transcription and expression of L-selectin ligands, which points out the crucial role of Fuc-TVII [16,17].

3.3. Ax cells possess Fuc-TVII protein

Intracellular immunoperoxidase staining with the rabbit polyclonal antibody raised against a murine Fuc-TVII peptide

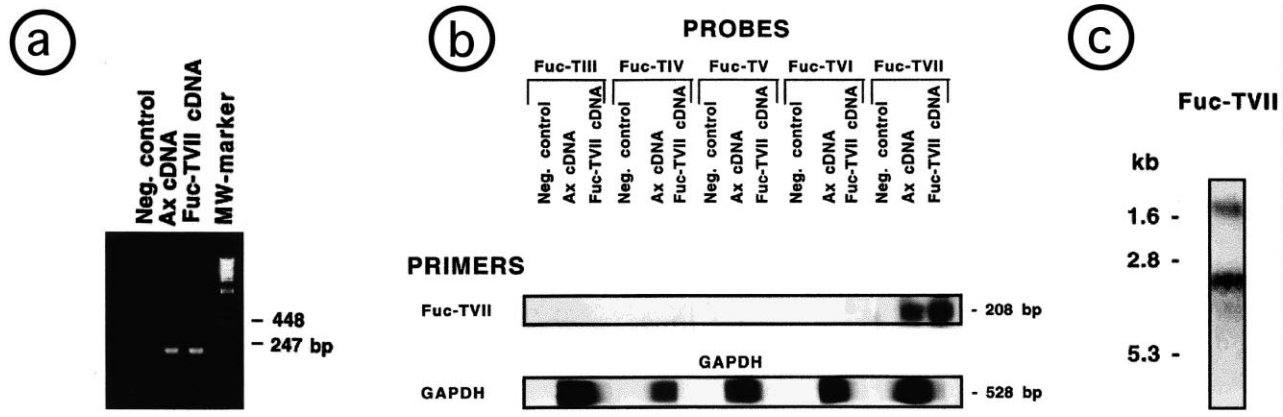

Fig. 2. Expression of α(1,3)fucosyltransferase Fuc-TVII in Ax high endothelial cell line. a: Detection of RT-PCR products by electrophoresis. b: The PCR product was probed with Fuc-TIII–Fuc-TVII cDNAs and only Fuc-TVII reacted. c: Ax cells have two Fuc-TVII mRNA transcripts as shown by Northern blot analysis. One representative experiment out of three is presented.

[12] showed that Ax cells expressed Fuc-TVII at the protein level (Fig. 3). CHO cells transfected either with Fuc-TVII or Fuc-TIV cDNA were cultured on chamber slides and used as positive and negative controls, respectively. The staining pattern in both the Ax and CHO-Fuc-TVII cells was perinuclear corresponding to the Golgi localization of this enzyme.

3.4. Ax cells have functional α(1,3)fucosyltransferase

After showing that Ax cells express sLex glycans and Fuc-TVII capable of synthesizing these epitopes we measured the α(1,3)fucosyltransferase activity as incorporation of [^{14}C]fucose from GDP-[^{14}C]fucose to exogenous sialyl-*N*-acetyllactosamine acceptor. The Ax cell lysate transferred 4 pmol of fucose to the acceptor in a 5 h assay. The structure of the reaction product was confirmed to be sLex NeuNAcα2-3Galβ1-4([^{14}C]Fucα1-3)GlcNAc by glycosidase treatments and paper chromatographic analysis.

3.5. Ax cells have α(2,3)sialidase and α(1,3)fucosidase activities

Besides the biosynthesis, it is also interesting to analyze the catabolism of sLex structures, as the balance of these two events yields the expression level of sLex at a given time. To analyze whether degradation of sLex by endothelial glycosidases would occur, we synthesized enzymatically the radiolabelled sLex-containing glycan NeuNAcα2-3Galβ1-4([^{14}C]Fucα1-3)GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc. The proximal *N*-acetyllactosaminyl unit was required for the protection of the otherwise very easily β-eliminated fucosyl residue. The β(1,6)GlcNAc branch was necessary to prevent the fucosylation of the proximal *N*-acetyllactosaminyl unit during the synthesis [18].

When 9.0 pmol of [^{14}C]-labelled sLex heptasaccharide was incubated with Ax cell lysate for 18 h, 73% of the original molecule became desialylated, representing neutral [^{14}C]-glycans and eluting with H_2O from the ion-exchange column (Fig. 4A). Concomitantly only 20% of the original structure remained intact and was eluted with acetic acid. To analyze further the nature of the neutral [^{14}C]-glycans i.e. whether the degraded reaction products were defucosylated, we subjected them to a paper chromatographic run (Fig. 4B). 6.0 pmol (corresponding to 67% of the original sLex-containing molecule, Fig. 4A) was desialylated and only 0.6 pmol (corresponding to 6% of the original sLex-containing molecule, Fig. 4A) was defucosylated as identified by the migration of the free radioactive [^{14}C]fucose on the paper (Fig. 4B).

Our data indicate that the primary route for sLex degradation by endothelial cells requires the desialylation of this glycan. Ax cells have a strong α(2,3)sialidase activity and also a minor α(1,3)fucosidase activity. Here, it is not known whether the defucosylated product originates from the sialylated or the neutral precursor. In vitro evidence with purified enzymes had already suggested that sLex is not degraded by α(1,3)fucosidases, but rather by α(2,3)desialylation [19].

To date, only fragmentary data are available on mammalian sialidases. These can be classified into three types; the

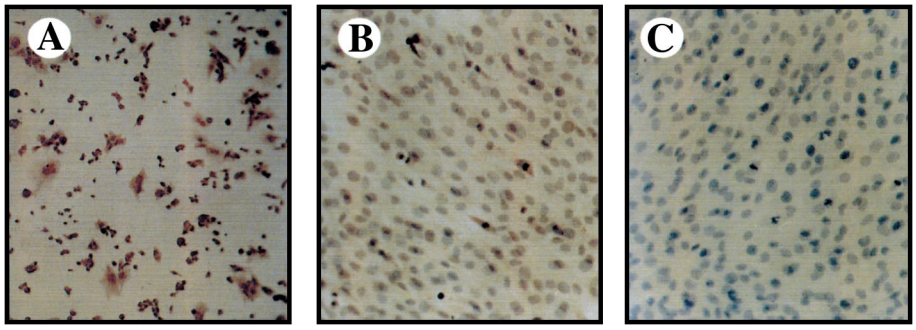

Fig. 3. Photomicrograph of immunoperoxidase stainings with Fuc-TVII antibody. A: Permeabilized Ax cells stained positively; B: positive control staining of Fuc-TVII; and C: negative control staining of Fuc-TIV transfected CHO cells.

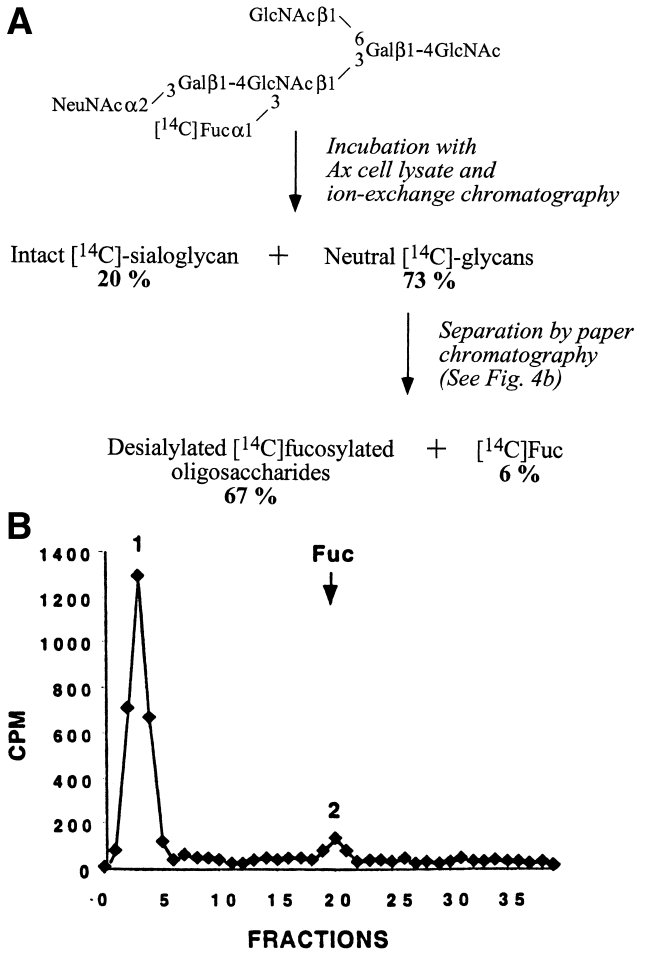

Fig. 4. Degradation of the radiolabelled sLex heptasaccharide by endothelial glycosidases. A: The degradation products were separated from the intact substrate by ion-exchange column, neutral products representing desialylated glycans and acidic ones sialoglycans. B: The nature of the glycans in the neutral fraction was further analyzed by paper chromatography. Two peaks could be seen: the desialylated fucose-containing glycan (peak 1), which migrated clearly slower, and the radioactive fucose monosaccharide (peak 2). The non-radioactive L-fucose was used as a marker.

cytosolic, lysosomal and plasma membrane-bound forms [20,21]. Currently the cellular localization and the type of endothelial sialidase(s) are not known. However, we have shown that the endothelial sialidase activity has a pH optimum around 4.0 suggesting that it is of lysosomal type ([22] and data not shown) and that desialylation most likely occurs in lysosomes after internalization from the cell surface.

Taken together, we show here that rat high endothelial cell line Ax has the functionally active enzymatic machinery to synthesize sLex from sialylated *N*-acetyllactosamines and degrade sLex to Lex glycans. The synthesis and degradation of 6-sulfated sLex in endothelial cells via these different pathways might be of crucial importance in the pathophysiological regulation of lymphocyte extravasation and should be considered as potential site for the development of anti-inflammatory approaches.

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