

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

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Received 30 March 1999; received in revised form 11 May 1999

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 $\alpha(1,3)$ fucosyltransferase Fuc-TVII, the enzyme responsible for the final fucosylation of sialyl-*N*-acetylglucosamine during sLex synthesis, and express sLex on the cell surface. Furthermore, these cells degrade sLex, primarily by desialylating it to neutral Lex epitopes by $\alpha(2,3)$ sialidase(s).

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Key words: Endothelium; Inflammation; $\alpha(1,3)$ fucosyltransferase; $\alpha(2,3)$ 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 $\alpha(2,3)$ sialic acid, $\alpha(1,3)$ 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 $\alpha(1,3)$ fucosylation of a sialyl-*N*-acetylglucosamine acceptor. Of the five cloned human $\alpha(1,3)$ 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*-acetylglucosamine 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 (NeuNAc α 2,3Gal β 1,4(Fuc α 1,3)GlcNAc β 1,3Gal β 1,4(Fuc α 1,3)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 x 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 μ l 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 TGAACCAAC 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 (TGAGCCCAAGGA TGCCCTTAGTG (no. 893–870 in X02231)). Each cycle for GAPDH 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 μ g 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

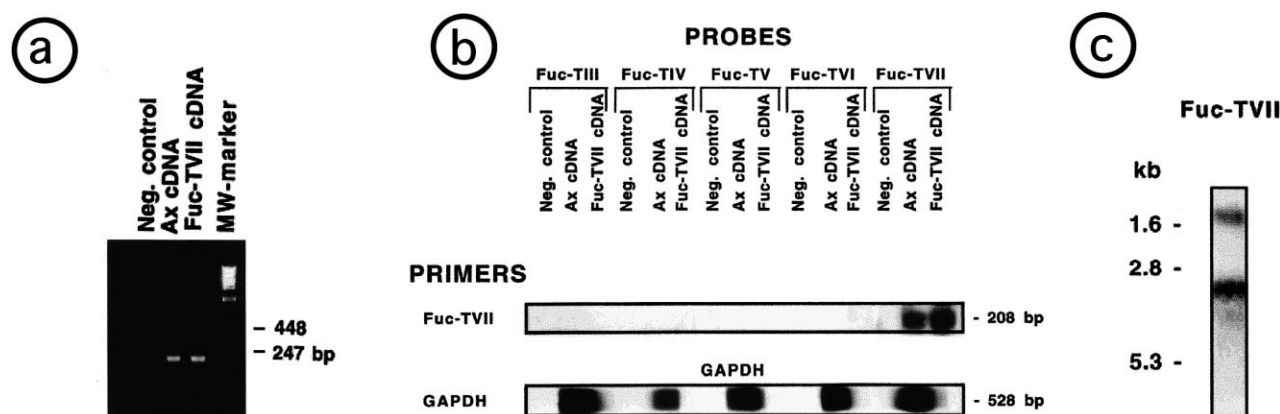


Fig. 2. Expression of $\alpha(1,3)$ fucosyltransferase Fuc-TVII in Ax high endothelial cell line. a: Detection of RT-PCR products by electrophoresis. b: The PCR product was probed with Fuc-TIII–Fuc-TVII cDNAs and only Fuc-TVII reacted. c: Ax cells have two Fuc-TVII mRNA transcripts as shown by Northern 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 $\alpha(1,3)$ fucosyltransferase

After showing that Ax cells express sLex glycans and Fuc-TVII capable of synthesizing these epitopes we measured the $\alpha(1,3)$ fucosyltransferase activity as incorporation of [14 C]fucose from GDP-[14 C]fucose to exogenous sialyl-*N*-acetylactosamine 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 NeuNAc α 2-3Gal β 1-4([14 C]Fuc α 1-3)GlcNAc by glycosidase treatments and paper chromatographic analysis.

3.5. Ax cells have $\alpha(2,3)$ sialidase and $\alpha(1,3)$ 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 NeuNAc α 2-3Gal β 1-4([14 C]Fuc α 1-3)GlcNAc β 1-3(GlcNAc β 1-6)Gal β 1-4GlcNAc. The proximal *N*-acetylactosaminyl unit was required for the

protection of the otherwise very easily β -eliminated fucosyl residue. The $\beta(1,6)$ GlcNAc branch was necessary to prevent the fucosylation of the proximal *N*-acetylactosaminyl 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 H₂O 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 $\alpha(2,3)$ sialidase activity and also a minor $\alpha(1,3)$ 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 $\alpha(1,3)$ fucosidases, but rather by $\alpha(2,3)$ desialylation [19].

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

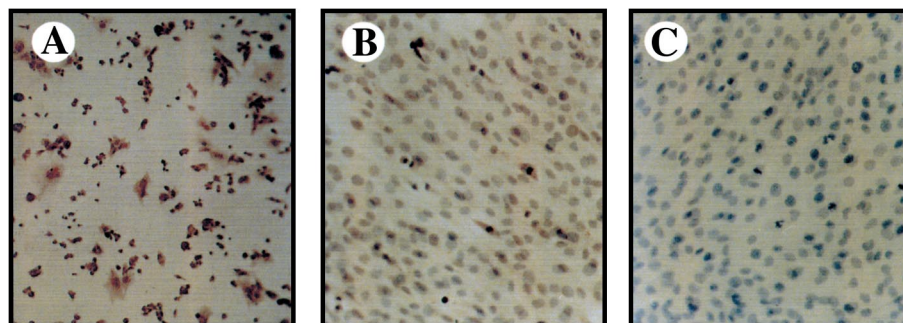


Fig. 3. Photomicrograph of immunoperoxidase stainings with Fuc-TVII antibody. A: Permeabilized Ax cells stained positively; B: positive control staining of Fuc-TVII; and C: negative control staining of Fuc-TIV transfected CHO cells.

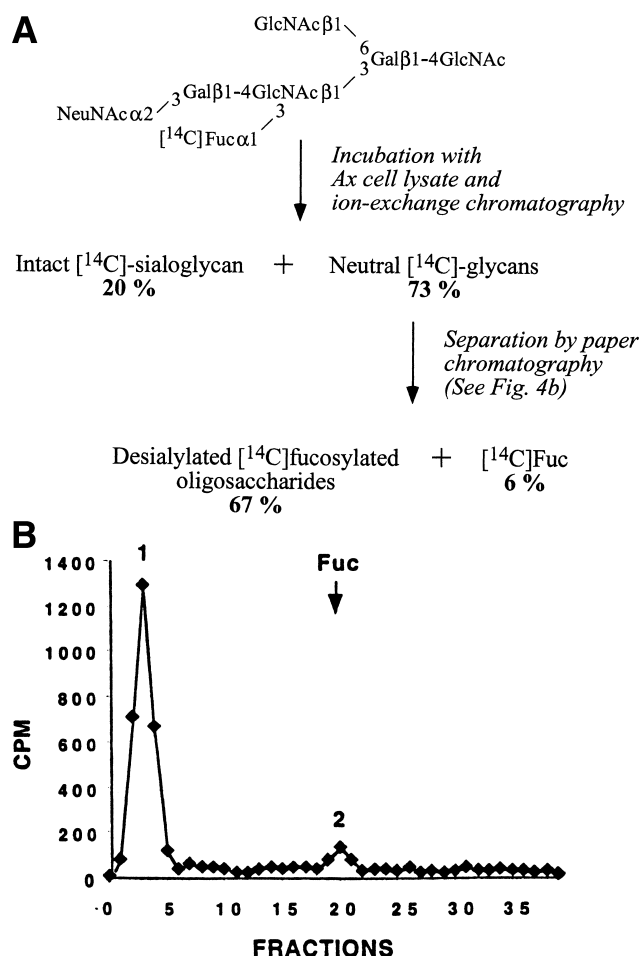


Fig. 4. Degradation of the radiolabelled sLex heptasaccharide by endothelial glycosidases. A: The degradation products were separated from the intact substrate by ion-exchange column, neutral products representing desialylated glycans and acidic ones sialoglycans. B: The nature of the glycans in the 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*-acetylglucosamines 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 consid-

ered as potential site for the development of anti-inflammatory approaches.

Acknowledgements: This study was supported by grants from Academy of Finland, the Technology Development Center of Finland, the Leiras Research Foundation, Paulo Foundation, Research and Science Foundation of Famos, University of Helsinki and the HUCH research grant and grant from the Ministry of Education to J.R.

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