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Introduction

The sialyl-Tn (sTn) antigen is a well known cancer-associated antigen, the expression of which is related to the prognosis of cancer patients. We aimed to isolate a human gene encoding an N-acetylgalactosamine-α2,6-sialyltransferase which synthesizes sTn antigen, and to characterize the enzyme. Degenerate primers encoding sialyl motifs were used for the polymerase chain reaction to amplify complementary DNAs prepared from RNAs of human pyloric mucosa with intestinal metaplasia, which abundantly expressed sTn antigen, followed by screening of full-length cDNAs using the amplified DNA fragment as a probe. We isolated two human cDNA clones, long-form (2.46 kb) and short-form (2.23 kb) cDNAs. The former encodes an active enzyme with a predicted 600 amino acid sequence. The latter, a splice-variant of the long-form, encodes an inactive enzyme. HCT15 human colorectal cancer cells stably expressing the long-form cDNA expressed sTn epitopes on O-glycans. The long form cDNA was considered to encode a human homologue of chick ST6GalNAc I for the following reasons:

1. The putative amino acid sequence showed greater homology to that of chick ST6GalNAc I (55%) compared to other sialyltransferases,
2. The putative amino acid sequence showed extraordinary long stem region that is a typical feature of chick ST6GalNAc I, and
3. The substrate specificity was very similar to that of chick ST6GalNAc I. In situ hybridization demonstrated that the localization of transcripts correlated well with that of sTn antigen in gastric cancer cells and Goblet cells in intestinal metaplastic glands. Thus, we determined that the long-form cDNA of the human ST6GalNAc I gene encodes the probable candidate for the human sTn synthase(s).

Key words: gastric cancer/intestinal metaplasia/sialyl-Tn antigen/sialyltransferase/ST6GalNAc

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Cloning and expression of human sTn synthase

So far, fifteen sialyltransferase (ST) genes, including the gene described in this study, have been cloned (Tsuji, 1996; Ishii et al., 1998; Kono et al., 1998; Lee et al., 1999). Sialylmotifs, which are conserved in all members of the ST family, were found by comparison of the amino acid sequences of the ST genes which were cloned earlier (Sjoberg et al., 1996; Tsuji, 1996). By the use of degenerate primers encoding sialylmotifs, additional STs have been successfully cloned (Kurosawa et al., 1996; Tsuji, 1996). Four of the 15 STs have been found to exhibit GalNAc α2,6-sialyltransferase activity, in that they can transfer NeuAc to O-GalNAc with an α2,6-linkage to an acceptor molecule. These have been named GalNAc α2,6-ST (ST6GalNAc I), Galβ1,3GalNAc-specific α2,6-ST (ST6GalNAc II), Neuα2,3 Galβ1,3GalNAc α2,6-ST (ST6GalNAc III), and ST6GalNAc IV (Lee et al., 1999). The ST6GalNAc I, II, and III genes have been cloned from chick (Kurosawa et al., 1994a), chick and mouse (Kurosawa et al., 1994b, 1996), and rat (Sjoberg et al., 1996), respectively. The substrate specificities of the recombinant enzymes directed by the three ST6GalNAc genes have been examined using oligosaccharides and O-glycosylated proteins as acceptor substrates (Kurosawa et al., 1994a,b, 1996; Sjoberg et al., 1996). Based on the in vitro specificities of the three recombinant enzymes, ST6GalNAc I is the most likely candidate for the sTn synthase(s) which is actually responsible for the expression of sTn antigen in native tissues.

To clone the gene encoding the human sTn synthase(s), which is probably homologous to the chick ST6GalNAc I gene, we initially performed Southern blot and Northern blot analyses using human genomic DNAs and human RNAs, respectively, using the chick ST6GalNAc I gene as the probe. However, we did not obtain positive bands that hybridized with the chick probe. This suggested that the human gene for ST6GalNAc I may not be homologous enough to hybridize with the chick probe. Therefore, the PCR method involving degenerate primers encoding two sialyl motifs was employed to amplify the respective genes (Kurosawa et al., 1996).

In the present study, we have successfully cloned the gene encoding a human ST6GalNAc I (hST6GalNAc I) and characterized the enzyme activity directed by the gene. The expression of the transcript for the hST6GalNAc I gene correlated well with the localization of sTn antigen in native tissues and cell lines. HCT15 cells stably transfected with the hST6GalNAc I gene expressed sTn antigens on mucins. These results strongly suggest that hST6GalNAc I is the most probable candidate for the human sTn synthase(s).

Results

Isolation of human cDNA clones which are candidates encoding sTn synthase

By use of the DNA fragment amplified with degenerate primers, two full-length cDNA clones with insert lengths of about 2.5 and 2.3 kbp, respectively, were isolated. Sequencing analysis revealed that the longer cDNA contains an ORF of 600 amino acids (DDBJ/EMBL/GenBank accession number is Y11339), and the shorter cDNA encodes a nucleotide sequence identical to that of the longer form except for the lack of a 234 bp segment, which is absent in the short-form cDNA, is boxed. The transmembrane domain is indicated by a dotted line. A possible polyadenylation signal is indicated by double underlines. The poly A tail is underlined with a thin line. The bar indicates the fragment used as an in situ hybridization probe, YI-ISHP2.

Fig. 1. (A) Nucleotide sequences of the two forms of cDNAs and their predicted amino acid sequences. The two sialylmotifs, i.e., L and S ones, are underlined. The 234-bp segment, which is absent in the short-form cDNA, is boxed. The transmembrane domain is indicated by a dotted line. A possible polyadenylation signal is indicated by double underlines. The poly A tail is underlined with a thin line. The bar indicates the fragment used as an in situ hybridization probe, YI-ISHP2.
bp segment at nucleotide positions 652–885 of the long-form (Y11339). The ORF of the short-form is not terminated by a frameshift, and extends to the termination codon, which is shared by the two forms (Figure 1A).

A part of the human genomic DNA encoding this gene was amplified by PCR, and sequenced to determine the exon–intron junction. In Figure 1B, the short-form cDNA can be seen as a splicing variant of the long-form lacking a 234 bp segment.

The putative amino acid sequence in the catalytic region (250 amino acid residues from the C-terminal end) encoded by the gene showed greater homology to mouse ST6GalNAc I (85%) (unpublished observations), mouse ST6GalNAc II (47.9%) (Kurosawa et al., 1996), mouse ST6GalNAc III (41.3%) (Lee et al., 1999), mouse ST6GalNAc IV (23.4%) (Lee et al., 1999), chick ST6GalNAc I (67.2%) (Kurosawa et al., 1994a), chick ST6GalNAc II (51.1%) (Kurosawa et al., 1994b), and rat ST6GalNAc III (43.5%) (Sjoberg et al., 1996) compared to other STs. We tentatively named this human gene hSTYI, and the putative enzymes encoded by the long-form and short-form transcripts of this gene will be referred to as hSTYI-L and hSTYI-S, respectively, in this paper.

Hydropathy profiles of the putative amino acid sequences of hSTYI-L and hSTYI-S, obtained by the Kyte and Doolittle method (Kyte et al., 1982), indicate that the ORFs encode a type II membrane protein with the typical topology of glycosyltransferases (i.e., a cytoplasmic tail of 15 amino acids, a transmembrane domain of 19 amino acids, a long stem region and a large catalytic region) (Figure 1A). hSTYI-S lacks a 78 amino acid fragment at positions 218–295 of hSTYI-L in the catalytic region. The stem regions of hSTYI-L and hSTYI-S are extremely long as estimated by Chou-Fasman analysis (Chou et al., 1974), as found with other glycosyltransferases. This feature of the long stem region is typical of chick ST6GalNAc I, but not of chick ST6GalNAc II or rat ST6GalNAc III (Kurosawa et al., 1994b, 1996).

**Detection of transcripts of the two forms, hSTYI-L and hSTYI-S, by RT-PCR in cancer cell lines and correlation with expression of sTn antigen**

Two human gastric cancer cell lines, MKN45 and MKN74, and three human colorectal cancer cell lines, WiDr, HCT15, and LSC, were examined for sTn expression by flow cytometric analysis using Mab TKH2 (anti-sTn). As can be seen in Figure 2, WiDr cells faintly expressed sTn antigen whereas LSC cells expressed substantial amounts of sTn antigen. In contrast, the other cells, HCT15, MKN45, and MKN74 cells, did not express sTn antigen at all (Figure 2A). As seen on RT-PCR, the three sTn-negative cell lines (HCT15, MKN45, and MKN74) expressed hSTYI-S transcripts, but not hSTYI-L transcripts (Figure 2B). In contrast, LSC cells and pyloric mucosa tissue, which were sTn-positive, expressed substantial amounts of hSTYI-L transcripts and very low amounts of hSTYI-S transcripts (Figure 2B). The above findings strongly suggest that only the long-form hSTYI-L transcript encodes an active form of sTn synthase, whereas the short-form does not encode an active enzyme.

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**Fig. 2.** Flow cytometric analysis and RT-PCR analysis for detection of the two forms of transcripts in cultured cell lines. (A) Flow cytometric analysis of sTn antigen expression. Dotted lines indicate the peaks stained without the first antibody, and solid lines indicate the peaks stained with TKH2 (anti-sTn) as the first antibody. (B) RT-PCR analysis. Molecular weight markers were run at the left. The long-form and short-form cDNAs were used as control templates, and the materials amplified by PCR from them were run at the right as size controls.
Stable expression of hSTYI-L and hSTYI-S cDNAs in transfection experiments of HCT15 cells

Three expression plasmid DNAs, pXLS, pXLA, and pXSS, were transfected into HCT15 cells. After 10 days of selection with geneticin, the HCT15 cells were analyzed by flow cytometry for the expression of sTn antigen. As shown in Figure 3, HCT15 cells transfected with pXLS, containing the sense long cDNA alone gave a positive peak with Mab TKH2, while HCT15 cells transfected with pXLA containing the anti-sense long cDNA, or pXSS containing the sense short cDNA, did not. Two groups of cells, transfected with pXLS and pXSS, respectively, were subjected to limiting dilution to obtain single transformant clones. Ten single transformant clones from each group were established, and the cloned cells which expressed pXLS were named HCT-sTn-L1 to -L10, and those which expressed pXSS were named HCT-sTn-S1 to -S10. Each single clone differed in the expression level of the respective transcript, which was determined by Northern analysis (data not shown). These single transformant clones were again analyzed by flow cytometry for sTn expression. Some of the HCT-sTn-L clones (e.g., HCT-sTn-L1 and HCT-sTn-L3) highly expressed the hSTYI-L transcript and were strongly stained with Mab TKH2 (Figure 3). In contrast, none of the HCT-sTn-S clones (e.g., HCT-sTn-S1 to -S10) gave positive staining with TKH-2, even though they expressed a substantial amount of hSTYI-S transcript (Figure 3).

As illustrated in Figure 3, wild-type HCT15 cells, HCT15 cells transfected with pXLA and pXSS, and HCT-sTn-S1 cells gave a positive staining peak with Mab FH-6 (anti-sLe^x). In contrast, HCT-sTn-L1 cells expressing hSTYI-L gave a completely negative peak for sLe^x but a positive peak for sTn antigen expression. This is the first direct evidence of reciprocal expression of the sLe^x and sTn antigens.

Substrate specificities of hSTYI-L toward various acceptors

Lysates of the wild-type HCT15 cells were assayed to determine their endogenous sialyltransferase activities toward various acceptors. The cell lysates of wild-type HCT15 and HCT-sTn-S1 cells exhibited very weak NeuAc-transfer activity only towards fetuin (i.e., ~0.5% of that of HCT-sTn-L1 cells) and undetectable activity toward acceptors other than fetuin (data not shown). Thus, we conclude that hSTYI-S encodes an inactive form of the enzyme.

Lysates of HCT-sTn-L1 cells exhibited strong NeuAc-transfer activity toward fetuin, asialofetuin, agalactosialofetuin, asialo-BSM, and asialo-glycophorin, and weak activity toward BSM (Table I, Figure 4A), but no activity toward α1-acid glycoprotein, asialo-α1 acid glycoprotein, benzyl-GalNAc, GM1, or asialo-GM1 (Table I). As demonstrated in Table I, the pattern of the relative activities of hSTYI-L toward various acceptors was quite similar to that of chick ST6GalNAc I, but not similar to that of the other two enzymes, i.e., chick ST6GalNAc II and rat ST6GalNAc III.

Polymers consisting of 2–7 units of Ala-Thr(GalNAc^α1)-Ala were also used as substrates. The polymer was found to be a very good acceptor substrate for hSTYI-L (Table I).

Determination of the linkage of NeuAc transferred to the GalNAc residue on fetuin

The O-linked carbohydrate structures of native fetuin are known to be NeuAc^α2,3Galβ1,3(NeuAc^α2,6)-GalNAc-Set/Thr and NeuAc^α2,3Galβ1,3GalNAc-Set/Thr. As shown in Figure 4A, hSTYI-L transferred NeuAc to three kinds of acceptors: native fetuin, asialofetuin, and agalactosialofetuin. Three kinds of sialidases were used to determine the linkage of [14C]NeuAc incorporated into fetuin with hSTYI-L. It is known that NDV-sialidase cleaves both α2,3- and α2,8-NeuAc linkages, and that NANase I cleaves only α2,3-NeuAc linkages. However, neither can cleave α2,6-NeuAc linkages. As can be seen in Figure 4B, treatment with either NDV sialidase or NANase I did not remove any [14C]NeuAc residues at all, and the radioactivity remained at almost the same level as in the mock-treated sample, although after treatment with both sialidases the radioactive bands shifted to positions corre-

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n.t., Not tested; n.d., not detected.
*Reported in Kurosawa et al., 1994b.
*Reported in Sjoberg et al., 1996.
*The activity against fetuin of each enzyme is presented as 100% activity.

Transcript levels of hST6GalNAc I on Northern hybridization

As illustrated in Figure 5, 5 μg of total RNA from the pyloric mucosa tissue (the source of the cDNA library), gave a single band of 2.5 kb size mRNA, corresponding to that of the long-form cDNA. We could not detect positive bands for any of the transcripts of the RNAs from spleen (lane 2), brain, or pancreas (data not shown) despite running larger amounts (20 μg) of total RNA in the gel. The RNAs of fundic gland mucosa with severe intestinal metaplasia, and duodenal mucosa, in which sTn antigen is strongly expressed, also gave the 2.5 kb positive band (data not shown). Twenty micrograms of total RNA from cultured cells were run on the gel (lanes 3–6). Only LSC cells, which strongly express sTn antigen, gave a 2.5 kb positive band, whereas the STn-negative cell lines did not (lanes 3–5). Figure 5A represents a 1% gel in which the two transcript forms, the long-form (2.46 kb) and short-form (2.23 kb), were not dissociated into two bands. In Figure 5B, a 1.6% gel was used to separate the two forms of transcript, HCT-stTn-L1 and HCT-stTn-S1 cells expressed abundant amounts of the long-form and short-form transcripts, respectively, although some of the short form transcripts in HCT-stTn-S1 cells were degraded.

Immunohistochemical and in situ hybridization analysis

STn antigen was detected immunohistochemically in two samples: noncancerous pyloric mucosa tissue with intestinal metaplasia and gastric cancer tissue. In the pyloric mucosa with intestinal metaplasia, goblet cells in the upper portion of the metaplastic glands expressed sTn antigen (Figure 6A). Mab TKH2 did not react with either the goblet cells in the bottom...
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1. Two sets of probes were prepared for in situ hybridization as described under Materials and Methods. The two antisense probes, YI-ISHP1 and YI-ISHP2, exhibited the same positive signals. As shown in Figure 6B, which is a serial section of Figure 6A, positive signals were detected in the cells located more closely to the bottom of the metaplastic glands in comparison with the localization of sTn-positive cells. The positive cells of the hST6GalNAc I transcript did not possess as many intracellular mucous droplets or mucous globules as the sTn-positive cells, as seen by in situ hybridization.

2. The gastric cancer cells immunohistochemically showed strong positive signals of sTn antigen on mucins accumulated in a mucin-lake (Figure 6D). hST6GalNAc I transcripts were positively detected by in situ hybridization in serial sections of cancer cells which secreted sTn-positive mucins into the mucin-lake (Figure 6E). The negative-staining with the sense probe, YI-ISHP1S, is shown in Figure 6C,F as a negative control.

**Discussion**

The biosynthetic pathway proposed for sTn antigen is presented in Figure 7, based on substrate specificities determined with the recombinant enzymes. ST6GalNAc I, most likely an sTn synthase as demonstrated in this study, may compete with Thomsen-Friedenreich antigen synthase(s) (T synthase(s)) for the Tn acceptor substrate. It may also compete with core 2 β1,6-N-acetylgalactosaminyltransferase (C2GnT) and α2,3-ST(s) for acceptor substrates. The T synthase(s) and α2,3-ST(s) involved in this pathway have not been identified at the molecular level yet.

Changes in carbohydrate structure are controlled by different mechanisms governing glycosyltransferase gene expression, such as transcriptional regulation, posttranscriptional regulation and alternative splicing (Harduin-Lepers et al., 1995; Kudo et al., 1995). Many splicing variant isoforms of the ST6Gal I gene have been found, and each of them is expressed in a tissue-specific manner (Harduin-Lepers, et al., 1995). In this study, cultured cells which did not express sTn antigen were found to express the short-form transcript, but not the long-form transcript. The sTn-positive cultured cells expressed both forms of transcripts. Future studies will explore the factors which regulate hST6GalNAc I expression in native tissues. It may be regulated not only through transcriptional regulation but also alternative splicing to express the active form of the enzyme.

Fig. 4. Assays to determine sialyltransferase activity towards fetuins, the linkage of the sialic acid transferred and the expression of the sTn epitope. The reaction mixtures of enzyme and substrate were separated by SDS–PAGE as described in Materials and methods, and then subjected to autoradiography (A, and the left of C) and to Western blot analysis (the right of C). (A) [14C]NeuAc-radioactive bands detected on autoradiography. Three acceptors were used: 1, fetuin; 2, asialofetuin; and 3, agalactoasialofetuin. L and S indicate the cell homogenates of HCT-sTn-L1 and HCT-sTn-S1 cells, respectively, which were used as enzyme sources. (B) The fetuin that had incorporated [14C]NeuAc was subjected to mock-treatment, or treated with NDV sialidase, NANase I or Vc NANase (from left to right). The residual radioactivity was detected by autoradiography after 5–20% gradient SDS–PAGE. (C) Asialo BSM was used as an acceptor substrate for the sialyltransferase assay. 1, After incubation with the cell homogenate of HCT-sTn-L1 (L) or HCT-sTn-S1 (S) as an enzyme source, asialo BSMs that had incorporated [14C]NeuAc were subjected to 5–20% gradient SDS–PAGE, and the radioactive bands were detected by autoradiography. 2. The same gel was subjected to Western blotting analysis with TKH2 (anti-sTn). 3, The HCT-sTn-L1 cell homogenate (40 µg), a four times larger amount than that used as the enzyme source, alone was analyzed for endogenous sTn antigen expression (L1). The homogenate of LSC cells (40 µg) was run on the right (LSC) as a positive control of sTn antigen expression.
enzyme resulting in the expression of sTn antigen in native tissues. However, the splicing out of the 234 bp segment in the short-form transcript does not comply with the rule of acceptor and donor site sequences of splicing (i.e., the GT-AG rule). Further study on this splicing form is required. Among the human cancer cell lines we examined, stable sTn expression was rare compared to Tn expression. This may be due to possible instability of this gene transcript by alternative splicing.

The sLe^x structure, a terminal epitope on complex carbohydrate chains, is carried mainly on mucins, and is a well known cancer-associated antigen. In leukemic cells, the sLe^x epitope is known to be carried on the termini of core 2-branched chains (Maemura et al., 1992). For the synthesis of a branch with the core 2 structure (Gal β 1,3(GlcNAc β 1,6)GalNAcα1-O-Ser/Thr), C2GnT transfers GlcNAc to the GalNAc residue in the Gal β 1,3GalNAcα1-O-Ser/Thr structure through a β 1,6-linkage. There have been some studies demonstrating that blocking C2GnT leads to the disappearance of the sLe^x epitopes. In vitro treatment with benzyl-GalNAc of cultured myelogenous tumor cells can block the synthesis of the core 2-branch on O-glycans in the cells, leading to decreased expression of sLe^x antigens and resulting in abolition of selectin-dependent adhesion (Kojima et al., 1992). If the sTn synthase is more predominant than C2GnT and T synthase in cells, the former would be expected to overcome the latter in the competition for acceptor substrates, resulting in termination of carbohydrate chain elongation and no synthesis of the core 2-branch, which would lead to the disappearance of the sLe^x terminal epitope. Therefore, reciprocal expression of sTn and sLe^x antigens might be predicted. In the present study, we have demonstrated for the first time that transfection of the hST6GalNAc I gene resulted in the expression of sTn epitopes and the disappearance of an FH6-reactive epitope, the sLe^x epitope, in HCT15 cells, probably due to the competition of hST6GalNAc I with C2GnT and T.
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The Golgi subcompartment in which hST6GalNAc I resides is not defined yet. It will be more carefully examined in the future as to whether both enzymes physiologically compete for substrate in the cells, since the glycosyltransferase overexpressed in the cells sometimes resides in nonphysiological Golgi subcompartment. In human leukocytes, sLex antigens expressed in the cells sometimes resides in nonphysiological for substrate in the cells, since the glycosyltransferase overexpressed in the cells sometimes resides in nonphysiological Golgi subcompartment. In human leukocytes, sLex antigens are expressed on myeloid and lymphoid cells, and play important roles in the rolling phenomenon of cells interacting with selectins on the endothelial cells of vessels (Rice et al., 1989). In a preliminary experiment, we observed that the long-form transcript for the hST6GalNAc I gene is also expressed at a substantial level in cultured human lymphocyte cell lines (data not shown). It will be interesting to determine whether or not the sLeα expression in myeloid lineage cells is regulated by the balance of the two enzymes: C2GnT which augments sLeα expression, and hST6GalNAc I which suppresses it. The sLeα antigens in cancer cells derived from digestive organs may contribute to their metastatic capacity by interacting with selectins on endothelial cells of vessels in distant organs. In fact, there have been reports of different prognosis in gastrointestinal cancer patients with sLeα-positive versus -negative cancers (Nakamori et al., 1993). Whether or not the sLeα expression in such cancer tissues is regulated by the two enzymes, i.e., C2GnT and hST6GalNAc I, remains to be determined.

The expression of sTn epitopes generally leads to underglycosylation of mucin molecules through termination of the elongation of O-linked sugar chains. Underglycosylated mucins are proposed to show dramatic changes in their tertiary protein structures and to expose the underlying tandem-repeat peptide backbone to immune cells. It was recently reported that the exposure of the peptide backbone of MUC1 mucin due to underglycosylation suppressed the proliferative responses of T-cells around cancer cells and led them to an anergy state (Agrawal et al., 1998). It was conjectured that this is the reason why cancer cells can escape attack by immune cells. In future experiments, we will examine whether or not underglycosylation occurs in HCT-sTn-L1 cells, and whether or not the underglycosylation of mucin molecules affects the immune response and the ability of tumor metastasis with the use of HCT-sTn-L1 cells.

It is known that intestinal metaplastic cells in the stomach originate from one or a few stem cells localized in the middle of the metaplastic glands. The stem cells produce daughter cells, which move to the luminal surface and reach the surface of the glands within 3 or 4 days. During movement to the top of the glands, they become more mature expressing some differentiation markers. Goblet cells also become more mature during movement, with many mucous droplets being present in the cytoplasm. It was noteworthy that the sTn antigen and the hST6GalNAc I transcripts were not completely colocalized in the same cells, but both were present in cells of the same cell lineage (i.e., goblet cells). sTn antigen was abundantly detected in the mucous droplets of mature goblet cells localized in the upper 2/3 of metaplastic glands. In contrast, the transcripts of hST6GalNAc I were mainly detected in the immature goblet cells containing small mucin vacuoles at the bottom of the glands. This finding is of interest as to the kinetics of the metabolic turnover of the transcripts and the carbohydrate antigens. It is possible that the turnover of transcripts is very rapid, but that the sTn antigen synthesized remains without degradation for several days and accumulates in cells of the goblet cell lineage during maturation.

In our previous paper (Kurosawa et al., 1994b; Table I), we reported that chick ST6GalNAc II also exhibits NeuAc-transferring activity to the acceptor agalactoasialofetuin with an α2,6-linkage, resulting in the synthesis of the sTn epitope, but its relative activity is very weak compared with that of chick ST6GalNAc I. Although some ST6GalNAc II activity may participate in sTn antigen synthesis in native tissues, we believe that hST6GalNAc I plays the major role. In the present study, we did not examine whether ST6GalNAc I transfers NeuAc to N-glycans in fetuin or not. However, hST6GalNAc I transferred NeuAc to the GalNAc residue of [Ala-Thr(Gal-NAc)-Ala]n polymer with an α2,6-linkage, and generated the sTn epitope on asialo-BSM and on the surface of transfected cells. These facts strongly implicate hST6GalNAc I as the most probable candidate for the human sTn synthase(s).

In the future, the hST6GalNAc I gene will be a very useful tool for elucidating the biological function of sTn antigen on a molecular basis, and for investigating the regulation of the expression of the aberrant sTn antigen as a cancer-associated antigen in native tissues.

Materials and methods

Cultured cell lines and monoclonal antibodies

The human colorectal cancer cell lines, WiDr and HCT15, and two human gastric cancer cell lines, MKN45, MKN74, were kind gifts from Dr. T. Kubota, Dept. of Surgery, Keio University School of Medicine, Tokyo, Japan. The human colorectal cancer cell line, LSC, was described in our previous study (Brockhausen et al., 1998). They are maintained in RPMI1640 (GIBCO-BRL, Rockville, MD) supplemented with 10% fetal bovine serum. Mab TKH2, anti-sTn (IgG) (Kjeldsen et al., 1988), was a kind gift from Dr. M. Tatematsu, Laboratory of Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan, and FH6, anti-sLeα (IgM) (Fukushi et al., 1984), was a kind gift from Dr. T. Irimura, Graduate School of Pharmaceutical Sciences, University of Tokyo.

Construction of a complementary DNA (cDNA) library

Nontumorous pyloric mucosa with intestinal metaplasia expressing abundant sTn antigen was obtained from the surgically resected stomach of a gastric cancer patient, and was subjected to the following experiments. Total RNA was prepared from the pyloric mucosal tissue by the guanidinium thiocyanate method (Chomczynski et al., 1987), and then used for cDNA library construction. Polyadenylated mRNAs were enriched with Oligotex-dT30 (Roche, Tokyo, Japan), and used for cDNA synthesis with a SUPERSCRIPT Choice System (GIBCO-BRL). The synthesized cDNAs were ligated with an EcoRI adaptor, and then inserted into a Lambda ZAP II vector (STRATAGENE, La Jolla, CA). The packaging procedure for the cDNA clones involved a Gigapack III Gold kit (STRATAGENE), according to the supplier’s manual.
Polymerase chain reaction (PCR) for cloning of a fragment encoding human sTn synthase

A degenerate primer set for PCR was designed based on the conserved amino acid sequences, which were found in the sialyl motifs common to the two chick ST6GalNAces, i.e., chick ST6GalNAc1 and II.

The cDNAs of the cDNA library described above were used as templates for the PCR amplification to obtain DNA fragment encoding human sTn synthase. PCR was performed with a degenerate primer set, 5′ primer, yi-1; 5′-AYGGNGGNATHYNAAAYAA-3′, and 3′ primer, yi-2; 5′-TGNAGNGCNTNAGCAGCAG-3′. The Y, H and N in the primer sequences indicate yt, h and n, respectively. The amplified PCR products were inserted into the p123T vector (MoBiTec, Göttingen, Germany). Twenty subclones in the p123T vector were sequenced by the dideoxynucleotide chain termination method using an ALF DNA sequencer (Pharmacia, Tokyo, Japan). We found four different sequences in the 20 clones. Three of them were known sequences already reported for human STs. One of them was a novel nucleotide sequence which, however, was homologous, 65.6%, to the corresponding region of the chick ST6GalNAc I gene.

Cloning of full-length cDNAs encoding a human sTn synthase

The 445 bp insert, that was found to be novel, was excised and used as a probe for hybridization to isolate full-length cDNA clones. We screened the cDNA library containing 1 × 109 independent recombinant phages constructed as described above, and isolated two distinct clones encoding a 2.46 kb insert (a long-form cDNA) and a 2.23 kb insert (a short-form cDNA), respectively. The inserts were subjected to nucleotide sequencing.

Determination of the partial genomic structure of the human sTn synthase gene

A primer set, yi3; 5′-AGGAGCATGCTCAACAAGGACG-3′ and yi4; 5′-AAGGCTGTGCACCTCTCGAC-3′, was employed to amplify the human genomic DNA fragment. The amplified fragment was subcloned into the p123T vector for nucleotide sequencing to determine the partial genomic structure of the human sTn synthase gene.

Reverse-transcription PCR (RT-PCR) analysis for detection of two forms of transcripts, i.e. long-form and short-form transcripts

Total cellular RNAs were prepared from various human tissues, which were obtained as surgically resected specimens, cultured cells of a series of cell lines, and transformant cells. The solubilized RNAs were treated with DNase I (GIBCO-BRL) at a concentration of 10 µg/ml at 37°C for 5 min to prevent contamination by genomic DNA. Complementary DNAs were synthesized with oligo(dT) primers from 5 µg of total RNA in a 20 µl total volume reaction mixture using a SUPERSCRIPT Preamplification System (GIBCO-BRL) for the first strand cDNA synthesis. After cDNA synthesis, the reaction mixture was diluted 50-fold with H2O and then stored at −80°C until use.

RT-PCR for detection of the respective transcripts was performed with ampli Taq Gold (Roche Molecular Systems, NJ) in a total volume of 50 µl containing 0.2 µM of a primer set, yi5; 5′-CCACCACCATGAGTCTGCTGCTG-3′ and yi4. The PCR buffer was supplied by the manufacturer (Roche). After preheating for 10 min at 94°C, 45 cycles of PCR were performed, each cycle comprising 1 min at 94°C, 1 min at 65°C, as the annealing temperature, and 2 min at 72°C.

Northern blot analysis

For Northern blot analysis, the method previously described in detail was employed (Kudo et al. 1995). A 1.85-kb fragment encoding the full-length open reading frame (ORF) of the long-form cDNA was labeled with [γ-32P]dCTP (Amersham, Buckinghamshire, UK) using a High Prime kit (Boehringer Mannheim, Tokyo, Japan), and used as a hybridization probe.

Transfection experiments to express the human sTn synthase gene in HCT15 cells

The full-length ORFs of the long-form and short-form cDNAs were subcloned into an expression vector, pCXN2 (Niwa et al., 1991). The plasmids obtained were named pXLS, which contains the long-form cDNA in the sense orientation, pXLA, containing the long-form cDNA in the antisense orientation, and pXSS, containing the short-form cDNA in the sense orientation. HCT15 cells, a human colorectal cancer cell line, were transfected with 10 µg of each of the expression plasmid DNAs by the electroporation method. The cells were selected in the presence of geneticin (G418; 0.6 mg/ml) (GIBCO-BRL) in RPMI 1640 (GIBCO-BRL) medium supplemented with 10% heat-inactivated fetal bovine serum. After 10 days exposure to geneticin, an aliquot of the cells was examined by flow cytometric analysis to determine the sTn antigen expression, and the rest of the cells were subjected to limiting dilution to obtain single transformant clones.

Sialyltransferase assay

A set of acceptors for the sialyltransferase assay were prepared as described in the previous study (Kurosawa, et al., 1994b). Polymers composing the 2–7 units of Ala-Thr(GalNAcct)-Ala were synthesized by organic chemical methods in Nishimura’s laboratory, Hokkaido University. The manuscript describing the detailed methods synthesizing the polymers is now under preparation. The method for the sialyltransferase assay was also described in detail in our previous paper (Kurosawa et al., 1994b).

The enzyme assays were performed in the presence of 50 mM MES buffer (pH6.0), 10 mM MgCl2, 5 mM CaCl2, 10 mM CMP-[14C]NeuAc (1.5 kBq) (Amersham), 1 mg/ml of acceptor substrate, and 15 µg of cell lysate as an enzyme source, in a final volume of 10 µl. After incubation at 37°C for 1 h, the reaction mixtures were subjected to 5–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for glycoproteins as acceptors, or to chromatography on HPTLC plates (Merck, Darmstadt, Germany) for benzyl-GalNAc and glycolipids as acceptors. Radioactivity incorporated into the products was quantified with a BAS2000 radio image analyzer (Fuji Film, Tokyo, Japan).

Identification of sialylated products

To determine the linkage of NeuAc incorporated into fetuin, three sialidases, Newcastle Disease virus (NDV) sialidase (Oxford Glycosystems Ltd., Bedford, MA), NANase I (NeuAc Linkage Analysis Kit; Glyco Inc., Novato, CA), and Vc-NANase
(Vibrio cholerae sialidase; Boehringer Mannheim), were employed to digest the incorporated [14C]NeuAc. The conditions for each sialidase treatment were described previously (Kurosawa et al., 1994b).

**Flow cytometric analysis**

The monoclonal antibodies used in this study were as follows. The expression of the sTn and sLea epitopes on the cell surface was examined by flow cytometric analysis using an Epics Elite (Coulter, Tokyo, Japan); 1 × 10⁶ cells were incubated with the respective first antibody (10 μg/ml) for 1 h on ice, and then washed twice with phosphate-buffered saline (PBS (pH 7.4)) containing 1% bovine serum albumin (BSA), followed by incubation with FITC-conjugated anti-mouse IgM or IgG. Then, the cells were washed again with PBS-BSA and finally subjected to flow cytometric analysis.

**Western blotting analysis**

The separated proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA) with Transblot SD cells (Bio-Rad, Richmond, CA). The membrane was stained according to the manual for the ECL Western blotting detection reagents (Amersham).

**Analysis by immunohistochemistry and in situ hybridization**

A human gastric mucosa sample was fixed with PBS containing 4% paraformaldehyde (pH 7.4) overnight at 4°C, and then embedded in paraffin (Histosec, Merck, Germany). Deparaffinized 4 μm serial sections were subjected to immunohistochemical analysis and in situ hybridization. The immunohistochemistry was performed by the same method as described previously (Ikehara et al., 1998), according to the manual for a Vectastain Elite ABC kit (Vector Lab., Burlingame, CA). The sections stained by the immunohistochemical procedure were counterstained with Meyer’s hematoxylin.

Two primer sets were designed for PCRs to amplify the fragments as in situ hybridization probes. One set of primers, yi5 and yi6, 5'-CCCTTCTTAAAAATGTCTGTGGCG-3', amplified the 166 bp fragment which is shared by both cDNAs, i.e. the long-form and short-form. The other set of primers, yi7; 5'-ACCAACAGCAGTCCACCACTAG-3' and yi8; 5'-TCTGAAGGCCTCATTTGCGG-3', amplified the fragment which is specific to the long-form cDNA. Each PCR product was subcloned in the pGEMT vector. The riboprobe labeled with digoxigenin (Dig RNA Labeling Kit; Boehringer Mannheim) were named YI-ISHP1, which is an antisense probe hybridizing to both forms of transcript, and YI-ISHP2, which is an antisense probe specific to the long-form transcript. The sense probes corresponding to each antisense probe were used as negative controls. The method for in situ hybridization was described in detail in our previous paper (Kurosawa et al., 1997).

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**Abbreviations**

sTn, sialyl-Tn; ST, sialyltransferase; NeuAc, sialic acid; O-GalNAc, O-N-acetylgalactosamine; ST6GalNAc I, GalNAc α2,6-sialyltransferase; ST6GalNAc II, Galβ1,3GalNAc-specific α2,6-sialyltransferase; ST6GalNAc III, NeuAc α2,3Galβ1,3Galα2,6-sialyltransferase; sLea, sialyl-Lewis a; sLeα, sialyl-Lewis x; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SD±PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSM, bovine submaxillary mucin; PF, parafomaldehyde; NDV, Newcastle Disease virus; Vc, Vibrio cholerae; NANase, sialidase; ORF, open reading frame; C2GnT, core 2 β1,6-N-acetylgalactosaminyltransferase.

**References**


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