N-Acetylglicosaminyltransferase III and V Messenger RNA Levels in LEC Rats during Hepatocarcinogenesis

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ABSTRACT

The LEC (Long-Evans with a cinnamon-like color) rat is a mutant of the Long-Evans strain which develops hereditary hepatitis and hepatoma with age. Activities and mRNA levels of N-acetylglicosaminyltransferase III and V (GnT-III and GnT-V, respectively) were determined during hepatocarcinogenesis in this rat using a LEA (Long-Evans with an agouti color) rat as a control. GnT-III activity in LEC rat liver increased after 30 weeks of age, at the stage of chronic hepatitis, to about 2.5-11.5 times the level in LEC rats aged 1-9 weeks. GnT-V activity in the LEC rat liver increased after 20 weeks of age, at the stage of acute hepatitis, to about 1.5-2.5 times the level in LEC rats of 1-9 weeks of age and then remained elevated. Both enzymes showed more dramatic increases in males than in females. The mRNA levels of the enzymes increased in proportion with the enzyme activities. Furthermore, GnT-III and GnT-V mRNAs were highly expressed in both cancer lesions and adjacent tissues. In one case of hepatoma with lymph node metastasis, GnT-III and GnT-V mRNA expression was much higher in the metastatic lesion than in the original cancer. GnT-III and GnT-V mRNAs in the original cancer lesion were similar to those in the cancer lesions of the other LEC rats. These results indicated that expression of GnT-III and GnT-V was induced by chronic liver damage and hepatocarcinogenic changes in the LEC rats.

INTRODUCTION

UDP-N-acetylglicosaminyl: β-mannoside β1,4 N-acetylglicosaminyltransferase (GnT-III) and UDP-N-acetylglicosaminyl: α-mannoside β1,6 N-acetylglicosaminyltransferase (GnT-V) are key enzymes in the branching of asparagine-linked oligosaccharides (1-3). GnT-III catalyzes the attachment of a N-acetylglicosaminyl residue to β1,4 mannose in the core region of N-glycans (see the assay procedure in Fig. 1). GnT-V catalyzes the attachment of a N-acetylglicosaminyl residue to the 6-position of the mannose α-1, 6 arm (see Fig. 1). High GnT-III activity has been observed in hepatic nodules of rat liver (4-7) and in other tumor cells (8, 9), and increased GnT-V activity has been shown to correlate with the metastatic potential of both rodent and human tumor cells (10, 11). The patterns of expression of these enzymes during carcinogenesis have not, however, been delineated. Recently we purified GnT-III from rat kidney and cloned its cDNA (12). We have also purified GnT-V from QG cells (human lung cancer cells) (13) and obtained some cDNA clones (see “Materials and Methods”).

The LEC rat is a mutant strain that was established from a closed colony of the Long-Evans strain. The mutant rats spontaneously develop acute hepatitis at 12-16 weeks of age, followed by chronic hepatitis, and eventually spontaneous hepatocellular carcinoma (14, 15). Recent reports have shown that the hepatic changes in LEC rats are closely associated with copper accumulation in the liver (16, 17). Consequently, the LEC rat is both a model for Wilson’s disease and a good experimental model for hepatocarcinogenesis. In this study, the expression of GnT-III and GnT-V was investigated in LEC rats during hepatocarcinogenesis.

MATERIALS AND METHODS

Animals. Animals were maintained at the Institute of Experimental Animal Science, Osaka University Medical School, and were killed under light ether anesthesia. Because LEC rats develop hepatitis followed by spontaneous hepatocellular carcinoma (14), livers from LEC rats were examined at various developmental stages after birth. The first symptom of acute hepatitis is jaundice, which is usually observed at 10-12 weeks of age after birth. High levels of serum glutamic pyruvic transaminase activities are maintained from this stage until 19-20 weeks of age. Most of rats (50–80%) die due to fulminating hepatitis at this stage. The other rats develop chronic hepatitis followed by spontaneous hepatoma after 30 weeks of age (18). In this study, the LEC rats were divided into 4 groups according to the clinical stage: first stage, 1–9 W (before hepatitis); second stage, 10–19 W (early acute hepatitis); third stage, 20–29 W (recovery from acute hepatitis); fourth stage, 30 W- (chronic hepatitis and hepatoma). Cancer lesions and uninvolved adjacent liver tissues were carefully excised from 52-week-old animals. They were stored at −80°C until used. The LEA rat, which is a sibling line of the LEC rat but does not develop hepatitis or hepatocellular carcinoma, was used as a control in some experiments.

Northern Blot Hybridization. Total RNA was prepared from livers stored at −80°C according to Chomczynski and Sacchi (19). Poly(A)+ RNA was purified with an oligo(dT)cellulose column (Pharmacia). RNAs were electrohoresed on 1% agarose gel and were transferred onto a Zeta-probe (BioRad) membrane by capillary action (20). The membrane filter was hybridized with 32P-labeled GnT-III or V cDNA at 42°C in hybridization buffer (20). About 1360 base fragment lying between HindIII sites was used as the GnT-III probe (12). The GnT-V probe was produced by the following methods. Partial amino acid sequences of tryptic peptides of purified GnT-V (13) were determined as follows: T-10, Thr-Pro-Trp-Gly-Lys; T-12, Asn-His-Gly-Ile-Leu-Ser-Gly; T-13, Asn-Ile-Pro-Ser-Tyr-Val; T-18, Val-Leu-Asp-Ser-Phe-Gly-Thr-Glu-Pro-Glu-Phe-Lys-His-Ala; T-21, Asn-Thr-Asp-Phe-Phe-Ile; T-23, Asn-Leu-Gln-Phe-Leu-Leu.

We selected T-18 and T-21 from these peptides and synthesized oligonucleotides predicted from the amino acid sequences. Underlined sections of the following peptide sequences were used for the design of oligonucleotide primer (Fig. 2). About 500 base nucleotide was obtained by polymerase chain reaction, using rat kidney cDNA as template. It was subcloned into Bluescript II KS+ (Stratagene) by EcoRl digestion, and then it was sequenced. The other amino acid sequences, T-10, T-12, T-13, and T-23, were found in the obtained clone (Fig. 2). This oligonucleotide was thought to be partial GnT-V cDNA and was used as a specific probe for GnT-V mRNA detection.

The filter was washed at 55°C with 2 × SSC (1 × SSC, 15 mM sodium citrate and 150 mM NaCl), pH 7.0 and 0.1% sodium dodecyl sulfate twice, 0.2% standard saline citrate, and 0.1% sodium dodecyl sulfate twice and then exposed to X-ray film (Kodak) with an intensifying screen for 6 days at −80°C.

Preparation of Crude Extract and Assay of GnT-III and GnT-V Activities. The liver samples and hepatoma tissues were homogenized in 4 volumes of 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 mM sucrose. After centrifugation at 900 × g for 10 min, the supernatants were collected and used as crude enzyme preparations. GnT-III and V activities were assayed using a fluores-

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2 The abbreviations used are: GnT-III, UDP-N-acetylglicosaminyl: β-mannoside β1,4 N-acetylglicosaminyltransferase III; GnT-V, UDP-N-acetylglicosaminyl: α-mannoside β1,6 N-acetylglicosaminyltransferase V; LEC rat, Long-Evans with a cinnamon-like color rat; LEA rat, Long-Evans with an agouti color rat; cDNA, complementary DNA; SSC, standard saline citrate.
GNT-III and GNT-V mRNA Levels in LEC Rats

GlcNAcβ1-2Manα1-GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA

GnT-III

GlcNAcβ1-2Manα1-GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-PA

GnT-V

Fig. 1. The assay procedure of GnT-III and GnT-V. GlcNAc, N-acetylglucosamine; Man, mannose; PA, 2-aminopyridine.

RESULTS

Age-related Changes in GnT-III and GnT-V Activities. The patterns of induction of GnT-III and GnT-V in hepatocarcinogenesis were investigated by examining the activities of these enzymes in the LEC rat liver at various ages. The LEC rat develops hereditary hepatitis at 12–16 weeks of age, and GnT-III activity was low before this stage. GnT-III activity increased after 30 weeks of age, at the stage of chronic hepatitis (Fig. 3). In contrast, GnT-V activity increased after 20 weeks of age, during the transition from acute hepatitis to chronic hepatitis (Fig. 4). For both enzymes, the increases were more remarkable in male rats than in female rats. In normal LEA rats, the enzyme activities were maintained at low levels.

Age-related Changes in GnT-III and GnT-V mRNA Expression. Northern blot hybridization analysis was used to follow mRNA expression. GnT-III mRNA was detected as a 4.7-kilobase band, and GnT-V mRNA was detected as 6.6- and 3.5-kilobase bands (Fig. 5). The level of GnT-III mRNA dramatically increased at 32 weeks of age, while GnT-V mRNA increased at 20 weeks of age and then was maintained at a high level. All 3 mRNA species were scarcely detectable before 12 weeks of age, although faint bands were observed at 8 weeks of age. The patterns of mRNA expression were consistent with the levels of enzyme activity.

GnT-III and GnT-V mRNA Expression in Cancer Lesions and Uninvolved Adjacent Tissues. The expression of GnT-III and GnT-V mRNA was investigated in hepatoma lesions and adjacent tissues (Fig. 6). High expression of GnT-III mRNA was observed in both cancer lesions and adjacent tissues. The level of GnT-III mRNA

Fig. 2. Schematic structure of a specific probe for GnT-V mRNA detection. Sense and antisense oligonucleotides were used as primer for polymerase chain reaction as described in "Materials and Methods." Sense 18: ggaattcGARCCNGARTTYAAYGC; Antisense 21: ggaattcATRAARAARTCNGTRTT (Single letter code: R = A or G; Y = C or T; n = A or C or G or T; n = A or C or G or T). T-18', T-13', T-12', T-21', and T-23' are consistent with the amino acid sequence of human GnT-V. T-18 and T-23' are consistent with those of the human GnT-V except one amino acid. H, restriction site of HindIII.

Fig. 3. GnT-III activity in LEC and LEA rat liver during hepatocarcinogenesis. The activity was determined according to the method described in "Materials and Methods." The tissue sources were total livers derived from LEC and LEA rats of various weeks of age. Bars, means from 1–4 male LEA rats (●), female LEA rats (○), male LEC rats (♦), and female LEC rats (□). Vertical line, SD of each group.

Fig. 4. GnT-V activity in LEC and LEA rat liver during hepatocarcinogenesis. The activity was determined according to the method described in "Materials and Methods." The tissue sources were total livers derived from LEC and LEA rats of various weeks of age. Bars, the mean for one to four male LEA rats (●), female LEA rats (○), male LEC rats (♦), and female LEC rats (□). Vertical lines, SD of each group.
GnT-III and GnT-V Expression in a Metastatic Lesion. Among five LEC rats aged 52 weeks, there was one case of hepatoma with metastasis at the paraaortic lymph node. Expression of GnT-III and GnT-V mRNA was investigated in this case (Fig. 7). The mRNA expression was remarkably higher in the metastatic lesion than in the original hepatoma or the adjacent tissue.

DISCUSSION

GnT-III catalyzes the addition of a bisecting N-acetylglucosamine in complex N-linked glycans. The enzyme activity is very low in normal rat liver and increases with malignant transformation (4, 6). Narasimhan et al. (4) reported that GnT-III was induced at the pre-neoplastic stage of hepatocarcinogenesis promoted by orotic acid. Herein we have shown that GnT-III activity in the LEC rat liver increased dramatically after 30 weeks of age, at the stage of chronic hepatitis (Fig. 3). This increase in activity resulted from an increase in expression of GnT-III mRNA (Fig. 5). Furthermore, high levels of the mRNA were detected both in cancer lesions and in adjacent tissues of 52-week-old LEC rats (Fig. 5). Pascale et al. (5) previously reported that high GnT-III activity was not detectable in tissues surrounding hepatic nodules in various rat models of hepatocarcinogenesis. This difference may be related to the cause of liver damage. The pathological changes in the LEC rat liver are diffuse (23), and even uninvolved tissues around the hepatoma can be considered preneoplastic lesions. In human chronic liver diseases, serum GnT-III activity increased at the stage of liver cirrhosis as well as hepatoma (24). In LEC rats, GnT-III was induced after chronic hepatitis. These phenomena suggest that the changes in GnT-III expression are related to liver regeneration or fibrosis.

GnT-V activity increased at 20 weeks of age, at the stage of acute hepatitis. This increase might be due to cytokines secreted at the site of acute inflammation. Nakao et al. (25) reported that interleukin 6 induced GnT-V in myeloma cells. Although high expression of GnT-V has been correlated with metastatic potential (10), GnT-V activity in
a case of LEC rat hepatoma with lymph node metastasis was not unusually high (data not shown) and no metastatic lesions were observed in two cases of hepatoma with high activities of GnT-V. Nonetheless, GnT-V mRNA expression in the metastatic lymph node was much higher than that in the original hepatoma (Fig. 7). GnT-III mRNA was also induced in the metastatic lesion. Because GnT-III and V mRNAs could not be detected in normal lymph node, activities of these enzymes were examined in lymphocytes. The activities were not high in comparison with those of other organs (data not shown). These results indicated that a clone with high activity of GnT-III and GnT-V moved to the lymph node and proliferated there.

The increases of GnT-III and GnT-V activities were more remarkable in male rats than in female rats. In LEA rats, the normal controls, no increase of GnT-III or GnT-V was observed in aging of either male or female rats. Furthermore, few differences have been observed in the incidence of hepatoma or neoplastic lesion between male and female LEC rats (18). Consequently, induction of GnT-III and GnT-V appears to be due primarily to hepatocarcinogenic changes. An augmenting effect of male hormones or a suppressing effect of female hormones could account for the observed differences in enzyme levels.

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