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Developmental Patterns of Galβ1,3(4)GlcNAc α2,3-Sialyltransferase (ST3Gal III) Expression in the Mouse: *In Situ* Hybridization Using DIG-labeled RNA Probes

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Sialic acids are key determinants for biological processes, such as cell-cell interaction and differentiation. Sialyltransferases contribute to the diversity in carbohydrate structure through their attachment of sialic acid in various terminal positions on glycolipid and glycoprotein (N-linked and O-linked) carbohydrate groups. Galβ 1,3(4)GlcNAc α2,3-sialyltransferase (ST3Gal III) is involved in the biosynthesis of sLe^x and sLe^a known as selectin ligands and tumor-associated carbohydrate structures. The appearance and differential distribution of ST3Gal III mRNA during mice embryogenesis [embryonic (E) days; E9, E11, E13, E15] were investigated by *in situ* hybridization with digoxigenin-labeled RNA probes coupled with alkaline phosphatase detection. On E9, all tissues were positive for ST3Gal III mRNA expression, whereas ST3Gal III mRNA on E11 was not detected throughout all tissues. On E13, ST3Gal III mRNA was expressed in different manner in various tissues. In this stage, ST3Gal III mRNA was positive only in the liver, pancreas and bladder. On E15, specific signal for ST3Gal III was detected in the liver, lung and forebrain. These results indicate that ST3GAl III is differently expressed at developmental stages of mice embryo, and this may be importantly related with regulation of organogenesis in mice.

Key words : Gal1,3(4)GlcNAc 2,3-Sialyltransferase, Embryogenesis

INTRODUCTION

Sialic acids are key determinants of carbohydrate structures involved in a variety of biological functions, and are transferred to the terminal position of the carbohydrate group of glycoproteins and glycolipids by a family of glycosyltransferases called sialyltransferases (Brandely *et al.*, 1990; Choo *et al.*, 1995; Paulson and Colley, 1989; Varki, 1992). To date, fourteen members of sialyltransferase gene family have been cloned and characterized (Kono *et al.*, 1996; Nakayama *et al.*, 1997; Tsuji, 1996). Four of them (ST3Gal I-IV) are considered to be involved in the formation of NeuAc α2,3Gal sequences in glycoconjugates of mammals through α2,3-linkage (Kono *et al.*, 1997; Tsuji, 1996). Mouse Galβ 1,3GalNAc α2,3-sialyltransferases (mST3Gal I and II), which synthesize the NeuAcα 2,3Galβ1,3GalNAc sequence of ganglioside as well as O-linked oligosaccharide of glycoproteins but show a different acceptor preference (Lee *et al.*, 1993 and 1994), exhibit the highest activity toward Galβ1,3GalNAc-(type III) and very low activity toward Galβ1,3GlcNAc-(type I), but negligible activity toward Galβ1,4GlcNAc-(type II) (Kono *et al.*, 1997). Mouse Galβ1,3(4)GlcNAc α2,3- and Galβ1,4(3)GlcNAc α2,3-sialyltransferases (mST3Gal III and IV) synthesize both NeuAc α2,3Galβ1,3GlcNAc- and NeuAcα 2,3Gal β1,4GlcNAc- sequences (Kono *et al.*, 1997). However, acceptor preference of mST3Gal III is different from that of mST3Gal IV; *i.e.*, type I serves as predominant acceptor for the former over type II which is much better acceptor for the latter (Kono *et al.*, 1997; Tsuji, 1996).

On the other hand, hST3Gal IV cloned from a human melanoma by lectin resistance selection is probably

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involved in the biosynthesis of the sialyl Lewis X (sLe^x) (NeuAc α2,3Galβ1,4[Fucα1,3]GlcNAc) determinant, which is a ligand for the E-, P-, and L-selectins known as cell adhesion molecules (Sasaki *et al.*, 1993). Although ST3Gal III and IV are likely to be related to sLe^x and sLe^a synthesis based on their substrate specificities (Sasaki *et al.*, 1993; Wen *et al.*, 1992), it is not clear which enzyme is mainly responsible for sLe^x synthesis. Recently, it was reported that the frequency of strong hST3Gal III (ST3N) amplification by PCR was significantly high in accordance with that of sLe^x staining in lung carcinoma, whereas the frequency of strong hST3Gal IV (STZ) was quite low compared with that of sLe^x staining (Ogawa *et al.*, 1997). This indicates that ST3Gal III may be responsible for the sLe^x synthesis.

The expression of sialyltransferases is tissue-specific and cell-specific and highly regulated during differentiation and proliferation, like in the case of other glycosyltransferases (Paulson *et al.*, 1989; Paulson and Colley, 1989). Since the main regulation of sialylation of glycoconjugates occurs at the level sialyltransferase expression, the comparison of enzyme expressions in tissue at different stage of development is of interest, indicating that it allows the identification of enzymes involved in the biosynthesis of stage-specific sialylated oligosaccharide structures.

Mouse embryogenesis and organogenesis, spanning the period from fertilization to term, have been characterized in detail by serial histological sectioning and scanning electron microscopy (Gilbert, 1988; Kaufman, 1992; Lawson and Biscoe, 1979; Theiler, 1989). To understand the developmental expression of ST3Gal III, we investigated the spatial and temporal expression pattern of ST3Gal III mRNA during the mouse embryogenesis by *in situ* hybridization. Our present study indicates that expression of ST3Gal III mRNA is developmentally regulated in tissue- and stage-specific manners.

MATERIALS AND METHODS

Preparation of Tissue

For histological observation, four prenatal [embryonic (E) days; 9, 11, 13, 15] B6C3F1 mice (Samyuk Laboratory Animals Inc., Korea) were used. To determine the age of embryos, the mean value of crown-rump (c-r) lengths of normal unfixed embryos in the same litter was calculated. Theiler's embryonic age scale and the corresponding mean c-r length are generally accepted method for determination of mouse embryonic age (Kaufman, 1992; Theiler, 1989). At least three embryos from different litters were studied at each embryonic age. Pregnant mice were kept in a constant dark-light cycle and allowed food pellets and water ad libitum. Embryos were dissected and immersed immediately into freshly prepared phosphate-buffered saline (PBS) containing 4% paraformaldehyde (pH 7.0). After fixation for 24 hours at 4°C, the embryos were washed in PBS, dehydrated through a graded series of ethanol washes, followed by cleared with xylenes and embedded in paraffin. Sections were cut at a thickness of 7 μm and mounted onto slides pretreated with aminopropyltriethoxysilane for adherence of sections.

Preparation of probe

For preparation of a probe for *in situ* hybridization assays, PCR was performed with a sense primer (5'-AGAGGTACCTTCGGAGCGACCGGGTCAG-3', containing a *Kpn* I site) and an antisense primer (5'-ATGAGTCGACGGTGACTGACAAGATGGC, containing a *Sal* I site), and the cloned mST3Gal III cDNA (Kono *et al.*, 1997) as a template. The PCR product (501 bp) was subcloned into the *Eco*R V site on pT7 Blue vector plasmid (Novagen, USA) and subsequently sequenced. The resulting cDNA sequences for mST3Gal III were comprised of 57 bp of the 5' untranslated region and 425 bp of coding region of mST3Gal III. A *Kpn* I-*Sal* I fragment (492 bp) of the cDNA for mST3Gal III was then subcloned into the same sites on pGEM-3Z (Promega, USA) and was linearized with *Kpn* I or *Sal* I. Digoxigenin-labeled antisense and sense RNA probes were synthesized by using T7 and SP6 RNA polymerase in the presence of digoxigenin-labeled UTP, respectively (Boehringer Mannheim, Germany).

In situ hybridization

DIG-labeled RNA *in situ* hybridization was done according to standard protocols with slight modifications (Kurosawa *et al.*, 1997). The sections were deparaffinized by placing slides in three changes of xylene for 10 min each and hydrated through a graded series of ethanol. All sections were treated in 0.2 M HCl at room temperature for 10 min and washed in PBS (pH 7.4).

The hydrated slides were treated with predigested proteinase K (10 μg/ml in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), fixed with 4% paraformaldehyde and acetylated with acetic anhydride. After ethanol dehydration, the sections were dried in air, and the tissue on the slides encircled with PAP-PEN (Daido Sangyo Co., LTD, Japan). Thirty microliters of hybridization solution (50% deionized formamide; 10 mM Tris-HCl, pH 7.6; 200 μg/ml yeast tRNA; 1×Denhardt's solution; 10% dextransulfate; 600 mM NaCl; 0.25% SDS; 1 mM EDTA, pH 8.0) containing 0.5 ng/μl DIG-labeled RNA probe were placed on each section and the sections were placed without coverslipping in an air-tight, humidified chamber containing 50% formamide and 2×standard saline citrate (SSC); the tissue sections were covered completely with the solution. The hybridization was carried out at 50°C overnight. After hybridization, all washes were performed

at 50μ consisting of two washes in 2×SSC, two in 0.2× SSC (each for 20 min). Finally, immunohistochemical detection of DIG-labeled RNA probes was carried out in accordance with instruction manual supplied by Boehringer Mannheim with some modifications: After washing the slides briefly (5 min) in buffer I (100 mM Tric-HCl, 150 mM NaCl, pH 7.5), the slides were incubated in buffer I with 10% normal bovine serum

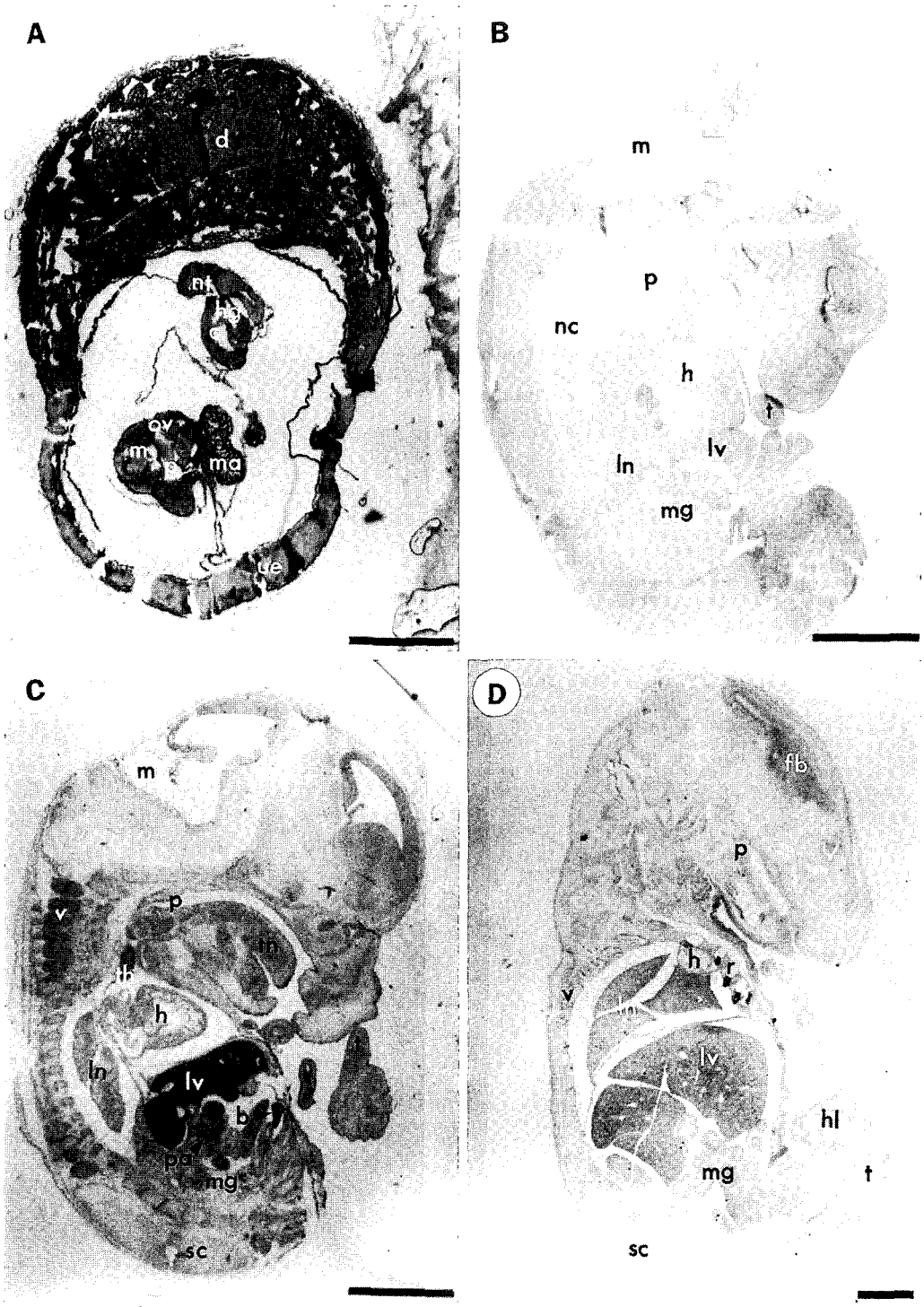

Fig. 1. Expression pattern of ST3Gal III mRNA in mouse embryos. ST3Gal III transcripts were detected by *in situ* hybridization with digoxigenin to sagittal sections of E9 (A), E11 (B), E13 (C), and E15 (D) mouse embryos. b, bladder; c, coelome; d, decidua; fb, forebrain; h, heart; hg, hind gut; hl, hind limb; ln, lung; lv, liver; m, myelocoel; ma, mandibular; mg, mid gut; nc, notochord; nt, nural tube; ov, optic vesicle; p, pharynx; pa, pancreas; r, rib; sc, spinal cord; t, tail; th, thymus; uc, uterine cavity; ue, uterine epithelium; v, vertebrae. The size of the scale bars is 1 mm. These are representative of 3 similar repeats.

(Sigma), 0.2% Tween 20 for 1 hr at room temperature. After 1:700 dilution of the anti-digoxigenin conjugate in buffer I containing 10% normal bovine serum, 50~100 μl of the antibody solution was applied to each tissue and incubated for 1 hr at room temperature in a humidified chamber using buffer I. Thereafter, the sections were washed for 15 min in buffer I and for additional 3 min in buffer II (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). Approximately 50~100 μl of color developing solution [per ml: 4.2 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 4.5 μl nitro blue tetrazolium (NBT)] was applied to each section, and slides were incubated at room temperature in the dark in a humidified chamber using buffer II. After 18 hrs, the reaction was stopped by washing the slides for 3 min in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, at room temperature. Finally, sections were mounted in mounting medium (10 mM Tris-HCl, 1 mM EDTA, 50% glycerine, pH 7.0).

RESULTS

We investigated the expression pattern of ST3Gal III gene during embryogenesis by *in situ* hybridization using DIG-labeled antisense RNA probes. In each embryonic day examined, the intensity of the hybridization signal, as evidenced by the alkaline phosphatase-induced purple color, was much stronger in the section hybridized with the antisense probe (Fig. 1A-D) than in the control sections hybridized with sense probe (Fig. 2). During embryogenesis, ST3Gal III mRNA was expressed in a restricted but complex pattern. On E9, ST3Gal III mRNA resulted in a strong signal localized throughout all parts of embryo (Fig. 1A). In contrast to this, on E11, hybridization signal was not detected in all organs of embryo (Fig. 1B). Interestingly, specific signal localized in various organs on E13, showing that high expression was detected in the liver, bladder and pancreas (Fig. 1C). On E15, the expression of ST3Gal III was observed in the lung, liver and forebrain (Fig. 1D). Table I summarizes the results of temporal and spatial expression pattern of ST3Gal III mRNA during embryogenesis.

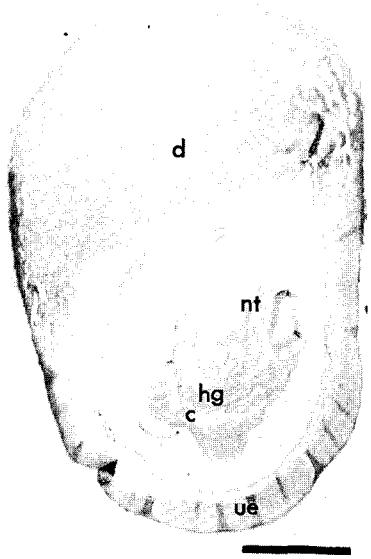

Fig. 2. Analysis of ST3Gal III sense probe on E9. The specificity of the reaction was shown by the negative staining observed when sense probe was used. d, decidua; nt, nural tube; hg, hind gut; c, coelome; ue, uterine epithelium. The size of the scale bar is 1 mm.

Table I. Summary of ST3Gal III mRNA during mouse embryogenesis

Tissues	E11	E13	E15
bladder	-	++	−
forebrain	-	−	+
liver	-	++	++
lung	-	−	++
pancreas	-	+	−

++, intensely positive ; +, moderately positive ; -, negative

DISCUSSION

It is well known that tissue-specific and developmentally regulated carbohydrate structures function as important mediators of cell-cell recognition (Bird and Kimber, 1984; Fenderson *et al.*, 1984; Rutishauser *et al.*, 1988; Sariola *et al.*, 1988) and differentiation (Nojiri *et al.*, 1986 and 1988; Tsuji *et al.*, 1983). One of the best characterized stage-specific embryonic antigens (SSEA-1) is a fucosylated structure [Galβ1,4(Fucα1,3)GlcNAc-R] that appears to be required for normal compaction of the mouse embryo at the 16-cell stage (Bird and Kimber, 1984; Fenderson *et al.*, 1984). Another example relevant to development involves the neural cell adhesion molecule (N-CAM) which mediates homotypic cell adhesion (Rutishauser *et al.*, 1988). During early development, N-CAM acquires a unique polysialic acid substituent on its N-linked oligosaccharides, and as the embryo matures, the length of these polysialic acid molecules is reduced.

In situ hybridization was used to determine the distribution of ST3Gal III mRNA in various embryonic stages during mouse embryogenesis. On embryonic day E9, ST3Gal III mRNA was expressed in all cells (Fig. 1A), suggesting that ST3Gal III is related to the early embryonic stages. On E13 and E15 during embryogenesis, ST3Gal III mRNA was detected throughout the liver, pancreas, bladder, lung and forebrain (Fig. 1C and D), suggesting that ST3Gal III expression may be involved in the embryonic processes as several other transcription factors. The brain is known to have a

particularly high content of gangliosides, and sialyltransferases have been reported in brain tissues of several animal species (Lee, 1995). However, it is not clear whether sialyltransferases is directly related to the brain development. In the forebrain of E15 mouse embryo during embryogenesis, ST3Gal III mRNA was positive (Fig. 1D), suggesting that ST3Gal III may play in brain development. Northern blot analysis from various mouse tissues showed that a large amount of ST3Gal III mRNA was highly expressed in brain, kidney, liver, colon, heart and spleen, and low in thymus and testis (Kono *et al.*, 1997). On the other hand, in embryonic day 15, high level of ST3Gal III mRNA was detected in brain and liver. Sialyltransferase so far cloned have been shown to exhibit remarkable tissue-specific expression, which is correlated with the existence of cell type-specific carbohydrate structure (Paulson, 1994). In general their expression appears to be regulated on the transcriptional level.

In summary, this result may have possible explanation; It is interesting that tissues specificity of ST3Gal III mRNA expression conserved among the different developmental stages. The results described here will provide further information for understanding the cellular control of recognition events mediated by carbohydrate groups in embryogenesis, development and differentiation.

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ABBREVIATIONS

PCR, polymerase chain reaction,; PB, phosphate buffer; EDTA, ethylene diaminetetraaceticacid; DEPC, diethylpyrocarbonate; TNE, Tris-HCl, NaCl, EDTA; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 20SSC,3 M NaCl, 0.3 M sodium citrate.

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