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[Short Communication]

The mRNA Expression of Neurocan, a Brain-Specific Chondroitin Sulfate Proteoglycan, in Neoplastic Mammary Glands in Mice

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ABSTRACT—In the experiment of mouse transforming growth factor alpha (TGFα) gene expression in mammary tumors, various sizes of amplified products by reverse transcriptase-polymerase chain reaction (RT-PCR) using mouse TGFα primers were detected in addition to a predicted size in four strains of mice. During the further analysis of these RT-PCR products in mouse mammary tumors, the transcript of neurocan gene was detected in the mammary tumor from SHN mice by the cloning and nucleotide sequence analysis after RT-PCR reaction using mouse TGFα primers. The 5'-nucleotide sequence of sequential 246bp in the amplified cDNA of 527bp was completely identical to a middle part of mouse neurocan cDNA sequence, one of the chondroitin-sulfate proteoglycan expressed in the nervous tissue.

INTRODUCTION

Transforming growth factor alpha (TGFα), which is a 50 amino acid polypeptide, was originally isolated from the conditioned medium of a murine sarcoma virus transformed cell line. TGFα shares approximately 30% homology with epidermal growth factor (EGF) and acts through binding with EGF receptors (Todaro *et al.*, 1980; Derynck, 1988). We have been investigating the endocrine participation in the effect of TGFα on normal and neoplastic mammary development (Mizuno *et al.*, 1994) based on the several reports which suggest that TGFα has functional roles in normal mammary gland and involves in mammary tumorigenesis (Liu *et al.*, 1987; Zajichowski *et al.*, 1988; Bates *et al.*, 1990; Matsui *et al.*, 1990; Snedeker *et al.*, 1991). In the previous study, we found the multiple sizes of the reverse transcriptase-polymerase chain reaction (RT-PCR) products using mouse TGFα primers in mammary tumors (Harigaya *et al.*, 1994a,b). The different sizes of cDNAs from a normal predicted size of TGFα cDNA in RT-PCR had not be dependent on the alternative splicing and those multiple sizes of PCR amplified products probably derived from the unknown mRNAs expressed in mammary tumors (Harigaya *et al.*, 1996).

In the present study, we attempted to determine the nucleotide sequences of some cDNAs amplified in RT-PCR reaction obtained from mammary tumors and unexpected cDNA, neurocan, which is one of the chondroitin-sulfate proteoglycan expressed in brain, was detected in the mammary tumor of SHN mice.

MATERIALS AND METHODS

Animals

C3H/He and SHN strains of mice maintained by the brother × sister mating in this laboratory were used. Females were retired after the 3rd lactation and were checked for palpable mammary tumors once a week. Three mice carrying mammary tumors were killed when the tumor sizes reached approximately 1 cm in a diameter. At autopsy, the portion of mammary tumor was immediately removed and stored at -80°C until use for the RNA extraction.

RNA isolation and RT-PCR

Total RNA was isolated from 100 mg of frozen mammary tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Reverse transcribed cDNA was synthesized with use of First-strand cDNA synthesis kit with cloned moloney murine leukemia virus reverse transcriptase (Pharmacia Biotec, Uppsala, Sweden). The target cDNA was amplified by PCR for 30 cycles (1 cycle = 94°C for 1 min, 55°C for 2 min and 72°C for 2 min) in a program temperature control system, Minicycler (Funakoshi, Tokyo, Japan) in the PCR buffer containing 2.5U *Taq* DNA polymerase (Takara, Kyoto, Japan), and 0.75 μM sense and antisense primers according to the mouse TGFα cDNA sequence (Vaughan *et al.*, 1992) as described previously (Harigaya *et al.*, 1994a).

Nucleotide sequence analysis

PCR-amplified cDNAs with mouse TGFα primers were ligated with pCRII vector and transformed into *E. coli* INVαF' competent cells in TA cloning kit (Invitrogen, San Diego, CA, U.S.A.). Transformed cells were cultured for 24 hr at 37°C in ampicilin- and Xgal-containing medium, and white colonies which contained cDNA inserted in lacZα-gene of pCRII plasmid were selected. Cloned pCRII plasmids were amplified and then subjected to the nucleotide sequence analysis. The nucleotide sequence of the cDNA was determined by the dye terminator cycle-sequencing method using a Model 373A DNA Sequencer of Applied Biosystems (Foster City, CA, U.S.A.) following the manufacturer's recommendations with a primer contained Sp6 or

T7 promoter sequence. Nucleotide sequence comparisons with the GenBank/EMBL/DDBJ DNA database were performed using BLAST program in Genomenet in Japan.

RESULTS AND DISCUSSION

In the RT-PCR experiment, multiple sizes of amplified products were detected in samples from mammary tumors in C3H/He and SHN strains as well as observed previously, while, a single band of RT-PCR product was also detected in one tumor sample of SHN strain (Fig. 1). In order to specify these PCR products, the nucleotide sequence analysis was performed with use of TA vector system for the construction of cDNA inserted plasmid. Plasmids of two colonies from a sample of SHN strain containing a single size of PCR product which is the same as a predicted size (349bp) of TGFα cDNA (Fig. 1, lane 3) were isolated and subjected first for *Eco*RI digestion analysis to estimate the length of inserted cDNA. Then, one of two plasmids which had the same length of inserted cDNA was determined to compare the sequence of mouse TGFα cDNA. This nucleotide sequence was identical to the mouse TGFα cDNA sequence except one nucleotide out of 349 bp which was adenine at 66 nucleotide corresponded to guanine in the sequence of the mouse TGFα cDNA reported previously (Vaughan *et al.*, 1992). However, this one nucleotide difference did not influence the amino acid sequence of TGFα protein (data not shown).

On the other hand, each ten colonies from a sample from mammary tumor of C3H/He or SHN strain (Fig. 1, lane 1 or 2) were randomly selected and subjected for plasmid isolation and *Eco*RI digestion analysis (Fig. 2). Four plasmids from a sample of C3H/He strain and three plasmids from a sample of SHN strain, in which the length of inserted cDNAs were less than 1kbp, were selected and applied for the further nucleotide sequence analysis. All of these sequences of plasmids which had different lengths of cDNAs from that of a normal TGFα cDNA had not similar sequences to mouse TGFα cDNA determined by a homology search using BLAST program in Genomenet DNA databases. In these plasmids, however, a part of one plasmid, sequential 246bp of 527 bp cDNA length, was found to be completely identical to the part of mouse neurocan cDNA sequence (Fig. 3). This cDNA was found to be amplified by the result of the same primer which annealed to both strands of cDNA. Sequences of other plasmids had not any high homology with known DNA sequences in DNA database.

Neurocan is one of the chondroitin sulfate proteoglycan which was expressed in brain and its cDNA sequence was reported in rats (Rauch *et al.*, 1992). The genomic structure was also reported in mice recently (Rauch *et al.*, 1995). The coding sequence of neurocan cDNA is large to 3.8kbp and the sequence determined in this study was only 246bp in the middle area of cDNA, nucleotide position at 1479 to 1724 and amino acids at 468 to 580 corresponded to the sequence reported previously (Rauch *et al.*, 1992). This region is corresponded to the middle of core protein area of neurocan (Rauch *et al.*, 1992). The sequence of cDNA obtained in the present experiment identical to a part of neurocan cDNA sequence is almost half size of 5' side of amplified cDNA. However, 3' half sequence was not found to be homologous to neurocan sequence or other any DNAs in database. This region had much GT repeat sequence.

Neurocan is thought to be an adhesion substance of neurons and glial cells and to be important in the neural development. The neurocan gene is highly expressed in brain tissue in mice and rats, but there are various forms of proteins in different stages of development (Rauch *et al.*, 1991; Matsui *et al.*, 1994). These several subtypes of neurocan proteins are possibly ascribed to alternative splicing or different post-translational processing (Rauch *et al.*, 1992). One possibility

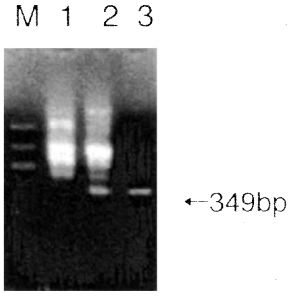

Fig. 1. Agarose gel electrophoresis of RT-PCR products from mammary tumors in C3H/He (lane 1) and SHN (lane 2 and 3) strains of mice. Lane M indicates DNA size marker.

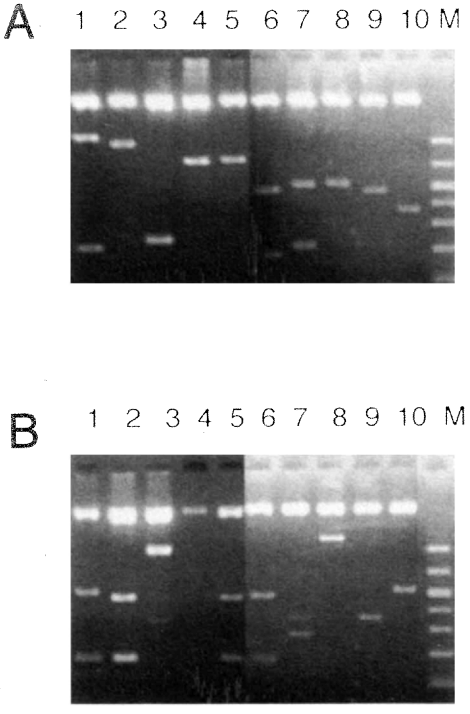

Fig. 2. Agarose gel electrophoresis of *Eco*RI digestion of ten isolated plasmids constructed from RT-PCR products from mammary tumor samples in lane 1 (panel A) and lane 2 (panel B) of Fig. 1.

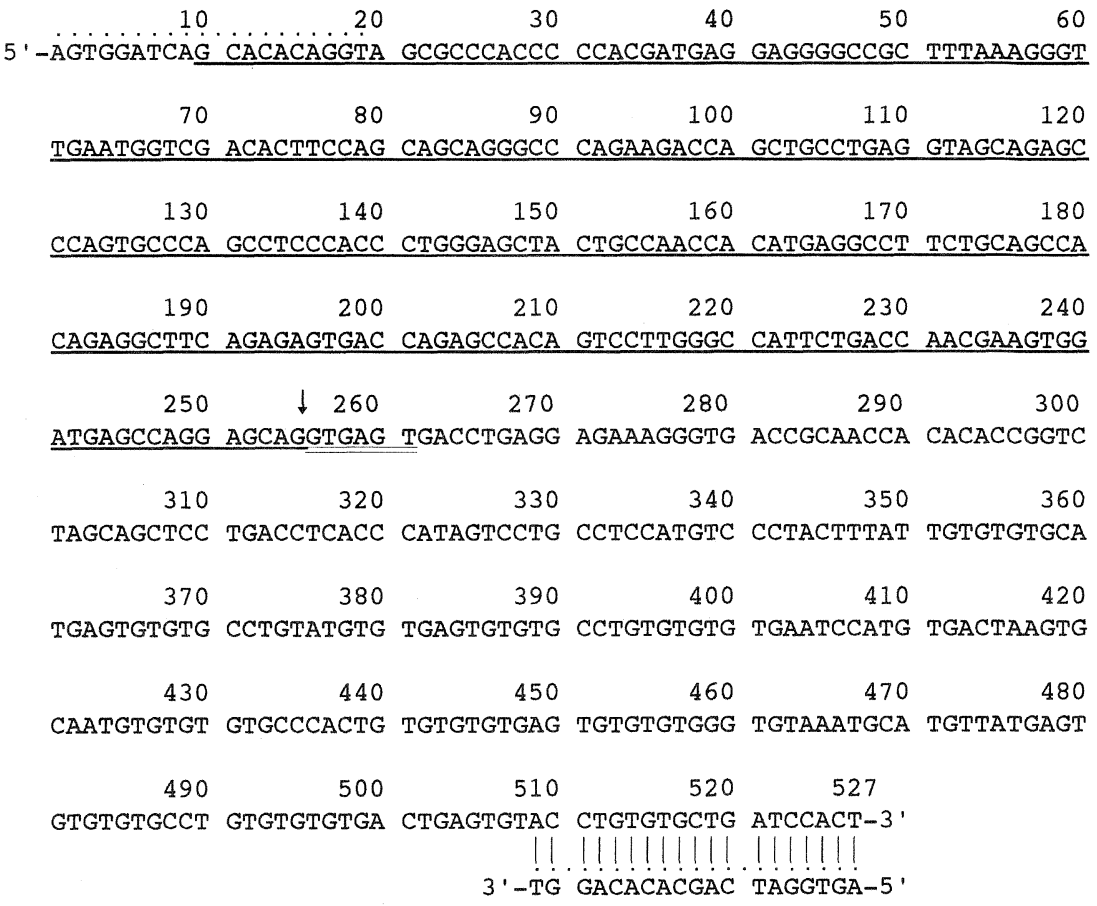

Fig. 3. Nucleotide sequence of cloned cDNA from mammary tumor containing partial mouse neurocan cDNA sequence. The cDNA was from plasmid No. 10 of panel B in Fig. 2. Nucleotides identical to mouse neurocan cDNA sequence (GenBank Accession No. X84727) are underlined. Double underlined nucleotides are identical to the intron 7 sequence and an arrow indicates the exon 7-intron 7 border suggested by the report of Rauch *et al.* (1995). Dotted nucleotides in both 5' and 3' ends indicate the primer sequence in this case. The same primer was annealed to both strands.

of the sequence obtained in this study is due to the occurrence of the alternative splicing of neurocan gene expression in mammary tumors so that the 3' half sequence of cDNA might be derived from the intron sequence which was spliced by different mechanism from that in normal brain tissue, because this region is identical to the sequence of exon 7-intron 7 junction reported by Rauch *et al.* (1995). Another possibility is that this cDNA might be from a prespliced type of mRNA, heteronuclear RNA. Since there is no report that neurocan gene is expressed in tumor tissues, this type of cDNA sequence might be a novel form of neurocan expressed in developing neoplastic tissues. It is necessary to determine the entire sequence of this neurocan cDNA expressed in mammary tumors and to clarify the function of neurocan in these tissues. Since the neurocan gene expression in mammary tumors was only detected presently in mammary tumor of SHN strain, further Northern blot analysis or screening of cDNA library in different strains of mice should be performed to determine whether strain difference of the neurocan expression is observed. Furthermore, other unknown mRNAs expressed in tumors which have similar sequences to mouse TGFα primers, but not any homologies to known DNAs, have to be elucidated in the future study.

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