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Original Article

Analysis of tenascin mRNA expression in the murine mammary gland from embryogenesis to carcinogenesis: an *in situ* hybridization study

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ABSTRACT The expression of tenascin gene during murine mammary gland development was analyzed by *in situ* hybridization with non-radioactive cRNA probes. The aim was to identify whether cells that synthesize tenascin are mesenchymal or epithelial. During embryogenesis, tenascin mRNAs were demonstrated in the epithelial cells of the mammary bud on the 14th and 15th day of gestation, and in the mesenchymal cells from the 14th day to the 17th day, at the epithelial-mesenchymal border of the growing bud. However, cells displaying tenascin mRNAs were not found beyond the bifurcation of the mammary sprout at the beginning of the branching morphogenesis. In post-natal development, tenascin mRNAs were demonstrated in mesenchymal cells surrounding end buds in juvenile mice, in mesenchymal cells surrounding the epithelial cells of plaques, in epithelial cells of the lactating mammary gland, in malignant epithelial cells and in the mesenchymal cells surrounding cancer nests. By immunohistochemistry, tenascin immunoreactivity was shown to have the same spatiotemporal distribution as that of tenascin mRNAs, but was observed to be restricted to the stroma, except in the lactating mammary gland where tenascin was demonstrated in the milk by Western blot. The present study thus showed that both epithelial and mesenchymal cells are sources of tenascin at different stages of murine mammary gland development.

KEY WORDS: *tenascin, in situ hybridization, murine mammary gland*

Introduction

Tenascin, now frequently referred to as tenascin-C (Erickson, 1993), is one of the ECM glycoproteins suspected to modulate epithelial-mesenchymal interactions that take place during the development of many tissues including the mammary gland (Chiquet-Ehrismann *et al.*, 1986; Inaguma *et al.*, 1988; Ekblom and Aufderheide, 1989; Sakakura, 1991; Beaulieu *et al.*, 1993). By immunohistochemical techniques, tenascin has been demonstrated in the murine mammary gland: in the mesenchyme surrounding the mammary buds of 14- to 16-day-old embryos and the endbuds of 3- to 4-week-old mice (Inaguma *et al.*, 1988), in the mesenchyme surrounding involuting mammary epithelium following lactation (Jones *et al.*, 1995), and in the stroma of mammary tumors (Inaguma *et al.*, 1988). Cells that synthesize tenascin have not yet been clearly identified, but they are thought to be exclusively mesenchymal (Inaguma *et al.*, 1988). In the human mammary gland, however, the combination of immunohistochemistry, *in situ* hybridization and *in vitro* studies has shown that both mesenchymal and epithelial cells synthesize tenascin (Lightner *et al.*, 1994; Ishihara *et al.*, 1995). The study of the mammary gland development in inbred mice developing mammary tumors is known to be one of the best systems for analyzing breast carcinogenesis *in vivo*. The developmental history of the murine mammary gland has not yet been screened for the expression of tenascin mRNAs *in situ* to identify the sites of tenascin synthesis. We therefore examined tenascin sources in the murine mammary gland by *in situ* hybridization techniques with non-radioactive complementary RNA (cRNA) probes.

Results

The expression patterns of tenascin mRNA in the murine mammary gland are summarized in Table 1. In the embryonic mammary gland, tenascin mRNAs were not detected in 13-day-old embryos (Fig. 1A), but were detectable from day 14 to day 17. In 14- and 15-day-old embryos, tenascin mRNA signals were found in both epithelial cells at the outer layer of the mammary bud

Abbreviations used in this paper: ECM, extracellular matrix; HAN, hyperplastic alveolar nodule; PBS, phosphate buffered saline; RT, room temperature; TBS, Tris-buffered saline.

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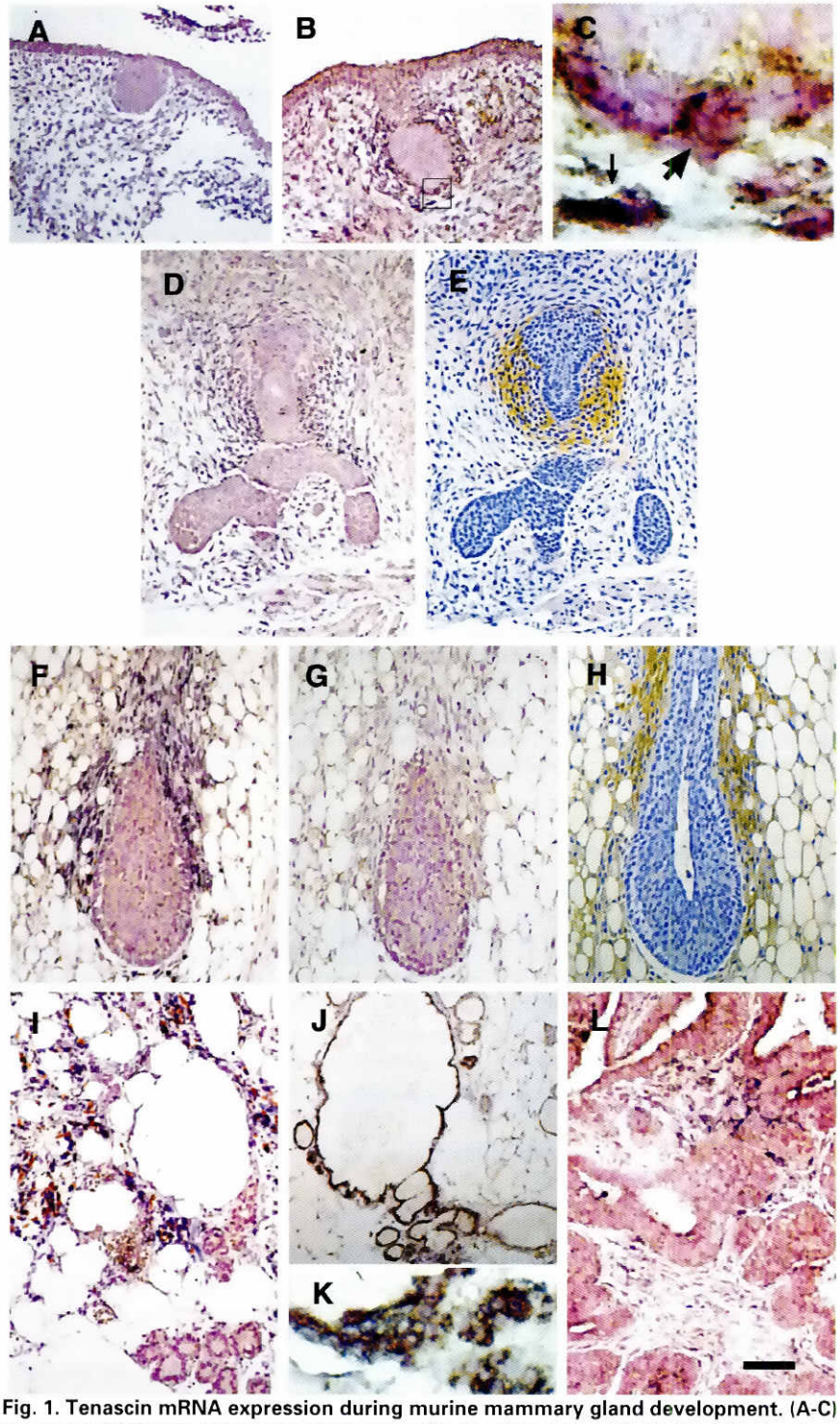

and mesenchymal cells surrounding the growing bud (Fig. 1B and C). From the 16th day, as the mammary bud was elongating and branching (17th day), the signals remained only around the stalk and the flank, not beyond the bifurcation, leaving the tips free; the signals were also found in the mesenchymal cells (Fig. 1D). In the juvenile mammary gland, the signals were localized in the mesenchymal cells around the neck region and the midregion of the endbud (Fig. 1F). A few signals were detected in the fat pad near the tip of the endbud. No signals were found in the epithelial cells of this stage. In the pregnant mice, tenascin mRNA signals were found, as shown in Figure 1L, in the mesenchymal cells of pregnancy-dependent tumors (plaque). In the lactating mammary gland, the signals were detected in the dense stroma surrounding mammary epithelial cells that did not undergo secretory transformation (Fig. 1I), and in some lactating epithelial cells (Fig. 1J and K). Although some tenascin immunoreactivity was detected in the involuting mammary gland, the mRNA signals were not observed (not shown). At the immunohistochemical level, tenascin immunoreactivity had the same distribution as that of tenascin mRNA, but was restricted to the mesenchyme (Fig. 1E and H), except in the lactating and the involuting mammary gland. In the lactating mammary gland, we observed some immunostaining in the lumen of some glands. We thought that tenascin may be secreted into the milk, and performed a Western blot analysis of the milk serum. As shown in Figure 3, we detected two tenascin bands of approximately 210 and 240 kDa.

In mammary tumors, tenascin mRNA signals were detected in both epithelial (Fig. 2A and E) and mesenchymal (Fig. 2D and F) cells. In both cases, cells carrying the signals were found at the epithelial-mesenchymal border. The expression of immunoreactive tenascin protein correlated well with the expression of tenascin mRNA, except that there was no immunoreactivity in epithelial cells (Fig. 2C). The ratio between epithelial and mesenchymal cells bearing tenascin mRNA tended to vary with the histological type (Table 1). A histologic classification of murine mammary tumors was done according to the

Fig. 1. Tenascin mRNA expression during murine mammary gland development. (A-C) *Longitudinal sections through the mammary bud.* **(A)** *No tenascin mRNA signals were detected at the 13th day of gestation.* **(B)** *On the 15th day signals were demonstrated around the growing mammary bud.* **(C)** *High-power view of an area in a box of (B). The thick arrow points to epithelial cells carrying tenascin mRNA, and the thin arrow points to mesenchymal cells also carrying the signals.* **(D)** *On the 17th day no signals were found beyond the bifurcation of the mammary sprout.* **(E)** *Tenascin immunostaining of the mammary sprout in (C) showing the same distribution pattern.* **(F-H)** *Tenascin mRNA expression during mammary duct elongation in juvenile mice. Longitudinal sections through the endbud.* **(F)** *Signals were detected in mesenchymal cells around the neck region and the midregion of the endbud.* **(G)** *Negative control incubated with the sense probe.* **(H)** *Same endbud as in (F,G) showing an identical pattern for tenascin immunostaining.* **(I-L)** *Tenascin mRNA expression in pregnant and lactating murine mammary glands.* **(I)** *On day 1 postpartum, the signals were found in mesenchymal cells surrounding mammary epithelial cells that did not undergo secretory transformation.* **(J)** *Lactating mammary epithelial cells showing the mRNA expression on day 7 of lactation.* **(K)** *High-power view of (J) showing epithelial cells bearing the signals.* **(L)** *Signals were detected in mesenchymal cells of a pregnancy-dependent mammary tumor (plaque). Bar in (L) indicates 100 μm for A,B,D-H,I and L; 10 μm for C; 250 μm for J; and 25 μm for K.*

TABLE 1

EXPRESSION OF TENASCIN (TN) mRNA IN MURINE MAMMARY GLAND

Glandular growth state	TN mRNA		TN protein*
	epithelial cells	mesenchymal cells	
Embryonic			
day 13	-	-	-
days 14 and 15	+	+	+
days 16 and 17	-	+	+
Juvenile	-	+	+
Adult	-	-	-
Pregnant	-	-	-
Lactating	+	-	-
Involuting	-	-	+
HAN	-	-	-
Plaque	-	+	+
Tumor**			
type A	±	+	+
type B	+	±	+

*detected in the stroma only. **in type A tumors, mesenchymal cells frequently expressed tenascin mRNA, while in type B tumors, the expression was predominant in malignant epithelial cells

system reported by Dunn (1953). Type A tumors are well-differentiated tumors with a fine uniform acinar structure. Tumors were classified as type B when solid cancer nests were formed. In the present type A tumors, the expression of tenascin mRNA was more frequent in mesenchymal cells than in epithelial cells, while in the type B tumors, the expression was predominant in malignant epithelial cells.

Next, we tried to clarify the expressed isoforms of tenascin, using a cRNA probe for the alternative splicing region of fibronectin type III-like repeats of tenascin (see Materials and Methods). There was no difference in the labeled patterns between probes for the larger tenascin isoforms and for the all isoforms (Fig. 2B). This indicates that the larger isoform is expressed in the developmental events.

Discussion

Previous studies have analyzed the distribution of tenascin immunoreactivity in the mouse mammary gland throughout its developmental history (Chiquet-Ehrismann *et al.*, 1986; Inaguma *et al.*, 1988; Sakakura, 1991). In the present study we have examined this distribution at both the protein and the mRNA levels to identify whether cells synthesizing tenascin are epithelial or mesenchymal. During embryogenesis, on the 14th and 15th days, tenascin mRNA-positive cells were not only mesenchymal but also epithelial. In the juvenile mice, the tenascin mRNA-positive cells were exclusively mesenchymal. In the lactating mammary gland, epithelial cells also showed the signals. These findings clearly indicate that both epithelial and mesenchymal cells produce tenascin during mouse mammary gland development, and that the expression of tenascin gene in these cells is spatiotemporally regulated. Our results parallel recent studies of the adult human breast showing that epithelial and mesenchymal cells in non-neoplastic and neoplastic tissues produce tenascin (Lightner *et al.*, 1994; Ishihara *et al.*, 1995). In addition, we have demonstrated, for the first time, that tenascin is secreted into the milk. The significance of the secreted tenascin into the milk needs further investigation.

In mouse mammary tumor, both mesenchymal and epithelial cells produce tenascin. The cellular sources of tenascin were related to the histologic types of mammary tumors, probably indicating different biological behaviors between these tumors. Type B solid mammary tumors were considered to be more malignant than type A tumors (Dunn, 1953). We found that epithelial cells in type B tumors predominantly synthesized tenascin. This may be compatible with the findings obtained from human breast

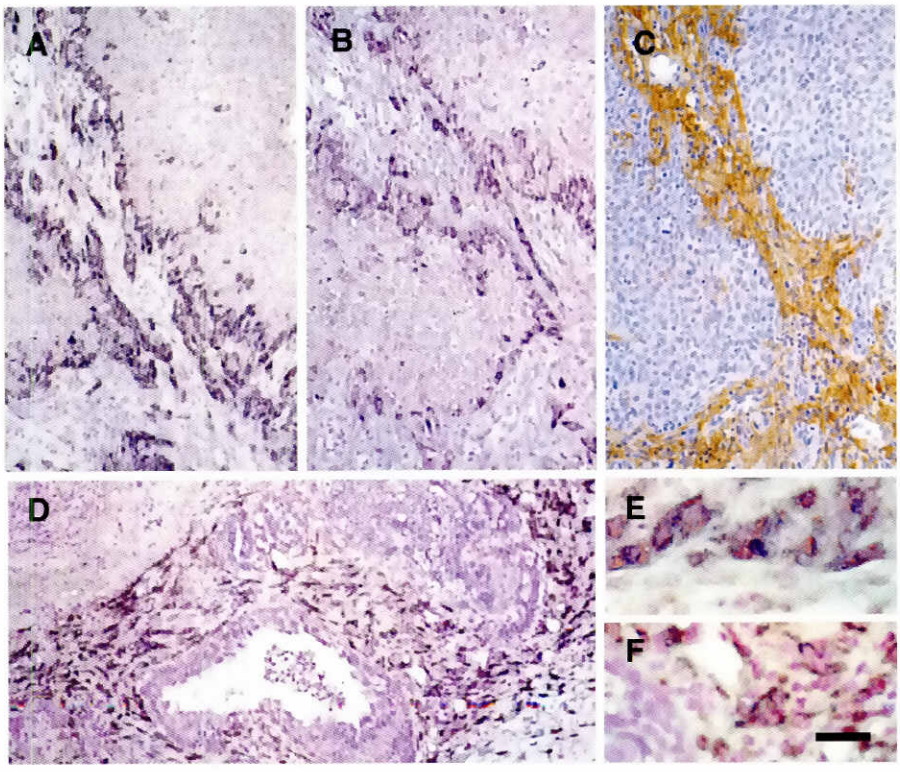

Fig. 2. Tenascin mRNA expression in murine mammary tumors. (A) *Type B cancer showing tenascin mRNA signals mainly in epithelial cells at the epithelial-mesenchymal border. A few signals are also detected in mesenchymal cells.* **(B)** *Same tumor stained with a probe for the alternative spliced region of tenascin mRNA.* **(C)** *Tenascin immunostaining of the same tumor in* **(A and B).** **(D)** *Type A tumor showing tenascin mRNA signals in mesenchymal cells surrounding cancer nests.* **(E)** *High-power view of the tumor in* **(A)**. *Malignant epithelial cells (top) are strongly stained, while the mesenchymal cells with long-shaped nuclei (bottom) are negative.* **(F)** *High-power view of the tumor in* **(D)**. *Mesenchymal cells (right) are positive for tenascin mRNA. Bar in (F) indicates 50 μm for A-D, and 25 μm for E-F.*

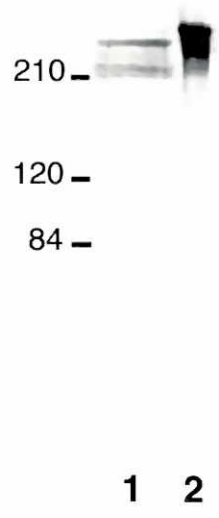

Fig. 3. Western blot analysis of milk serum. *Tenascin was detected by immunoblots with a rabbit anti-human tenascin polyclonal antibody. Molecular weight markers are shown to the left. In* **(lane 1)**, *5 µl of milk serum was loaded, and two bands of approximately 210 kDa and 240 kDa were detected.* **(Lane 2)** *was loaded with 0.1 µg of human melanoma tenascin, and shows a higher band, over 250 kDa, of human tenascin than that of mouse tenascin.*

cancers that the tenascin positivity of cancer cells was closely related to a poor outcome of the patients (Ishihara *et al.*, 1995).

Tenascin isoforms resulting from the alternative splicing of the fibronectin type III-like domain have been described in mice (Saga *et al.*, 1991). However, no analysis of tenascin splicing variant expression in the murine mammary gland has been reported to date. The largest isoform which includes the alternatively spliced fibronectin-III repeats was reported to be predominant in mouse kidney (Saga *et al.*, 1991; Weller *et al.*, 1991) and rat lung (Young *et al.*, 1994) embryogenesis, whereas the smallest isoform was predominant in mouse gut embryogenesis (Saga *et al.*, 1991; Weller *et al.*, 1991). Our data indicate that the larger isoform is expressed during murine mammary gland development. The alternative splicing of tenascin gene transcripts could be developmentally regulated and its regulation may be different among the tissues.

Materials and Methods

Animals

Mammary tissues were collected from GRS/A mice. These mice were originally obtained in 1976 from the Netherlands Cancer Institute-Antoni van Leeuwenhoekhuis Hospital, Amsterdam. They are kept at the animal section of our University. The day of the vaginal plug was considered as day 0. Mammary tissues were removed from 13- to 17-day fetuses, from 3- to 4-week-old, fully matured 12-month-old virgin, midpregnant, lactating (9 days of lactation), and involuting (2 days and 7 days after withdrawal of the babies) female mice. Preneoplastic HAN, plaques (at midpregnancy and on day-1 postparturition) and mammary tumors were isolated from breeders.

Preparation of cRNA probes

Antisense and sense cRNA probes were prepared by the *in vitro* transcription of mouse tenascin cDNA (Saga *et al.*, 1991), using a digoxigenin RNA labeling kit (SP6/T7; Boehringer Mannheim, Mannheim, Germany) as previously reported (Tsukamoto *et al.*, 1991). We used two kinds of cRNA probes to examine tenascin isoforms. The 6th-11th fibronectin type III-like repeats (named A1, A2, A4, B, C and D) of mouse tenascin are alternatively spliced sites. Tenascin mRNA including these sequences generates tenascin isoforms of larger molecular weight. In the present study, a probe derived from the cDNA encoding these repeats, designated FNAE by Tsukamoto *et al.* (1991) was used alone to detect larger isoforms. The probes for other regions were used as a mixture to detect mRNAs for all isoforms of tenascin.

In situ hybridization

The preparation of mammary tissues, *in situ* hybridization method and color development were previously described by Ishihara *et al.* (1995). Tissue sections were treated with protease K for 10 min.

Collection and Western blot analysis of mouse milk

Milk was collected at the 7th day of lactation after an overnight isolation from the babies, and stored frozen at -70°C. Milk was centrifuged at 12,000g, at 4°C for 15 min, and the top lipid layer was discarded. The aqueous layer was collected for analysis. Electrophoresis was accomplished on a 7% gel according to the method of Laemmli (1970). The milk serum was mixed with an equal volume of 2x sample buffer, and 10 µl of the mixture was applied on the gel. Purified human melanoma tenascin (Otsuka Assay Co., Tokushima, Japan) was used as a positive control (0.1 µg/lane). The proteins were then transferred onto a PVDF Immobilon membrane (Millipore Corp., Bedford, MA, USA). The membrane was blocked in 10 mM Tris-HCl and 150 mM NaCl (TBS) with 1% skim milk at RT for 2 h, followed by incubation in a 1:1000 dilution of rabbit anti-human tenascin polyclonal antibody at RT for 2 h. The membrane was washed three times in TBS with 0.3% skim milk, and then incubated in a 1:400 dilution of anti-rabbit peroxidase-conjugated goat IgG (MBL, Nagoya, Japan), followed by three washes in TBS with 0.3% skim milk. Color development was done with a diaminobenzidine/H_2O_2 solution.

Immunohistochemistry

After deparaffinization and rehydration in a graded series of ethanol, the sections were incubated in 0.6% H_2O_2 in methanol for 30 min to block the endogenous peroxidase activity. They were then washed in 10 mM PBS and incubated in 5% normal goat serum for 30 min. A rabbit anti-human tenascin polyclonal antibody (diluted 1:500) was applied to the sections for 1 h at RT (in negative control sections the antibody was replaced with PBS). This incubation was followed by three washes in PBS for 10 min. The sections were then treated with a goat anti-rabbit peroxidase-labeled antibody (MBL) for 30 min, and washed in PBS three times for 5 min each. The color development was done with a vectorstatin DAB-kit (Vector Laboratories, Burlingame, CA, USA).

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