Changes in Tenascin-C Isoform Expression in Invasive and Preinvasive Breast Disease

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ABSTRACT

Tenascin-C (TN) is an extracellular matrix protein that is expressed at low levels in normal adult tissue but is highly expressed around many tumors including breast carcinoma. TN exists as multiple isoforms generated through alternative splicing, and these isoforms have different effects on cell growth and migration. This study has analyzed in detail the pattern of TN isoform expression in benign, preinvasive, and invasive breast lesions using reverse transcription-PCR and Southern blotting. Significant differences in the profile of TN isoforms were identified. Although all tissues expressed the fully truncated TN, expression of two additional isoforms, one containing exon 16 (TN16) and one containing both exons 14 and 16 (TN14/16), were significantly associated with the invasive phenotype (P < 0.001). A subset of ductal carcinoma in situ (DCIS) cases were also found to express these isoforms, which may be indicative of a high risk of invasion in these lesions. Expression of these isoforms correlated with the presence of TN protein in the stroma in place of or in addition to basement membrane TN. Immunohistochemistry and in situ hybridization confirmed the production of exon 14-containing higher molecular weight isoforms by stromal fibroblasts in malignant tissue and both periducital fibroblasts and residual myoepithelial cells in DCIS. Although no evidence of tumor cell synthesis of TN was detected in the tissues, two highly invasive breast cancer cell lines (MDA-MB 231 and MDA-MB 468) were found to produce TN in contrast with tumor cells with a lower invasive capacity (MCF-7 and T47D).

These results demonstrate for the first time that specific TN isoforms are expressed in invasive breast carcinomas and that these isoforms are identified in a subset of DCIS and suggest that detection of TN16 and/or TN14/16 may be used as a predictor for invasion. Functional studies are now essential to establish the effect of these isoforms on tumor behavior and evaluate whether they will provide appropriate targets for therapeutic intervention.

INTRODUCTION

Cellular interactions with the ECM are vital for the transduction and integration of signals from the microenvironment. Such interactions occur through specific cell surface receptors and have a profound influence on cell behavior including modulation of cell growth, differentiation, migration, and survival (1). Exposure of cells to different matrix components can elicit very different effects (2), and subtle structural changes in the ECM induced by local proteolysis can also influence cell behavior (3). Thus, matrix composition and structure plays a central role in maintaining normal cellular homeostasis. It has long been recognized that the stroma around a malignant tumor differs from normal ECM. One change that is seen in the stroma of many solid tumors is overexpression of the ECM glycoprotein TN (4–6). In breast carcinomas, high expression of TN has been related to poor prognosis (7), and local and distant recurrence (8), and expression in DCIS has been suggested to predict invasion (9, 10).

The action of TN is complex. It can influence cell behavior directly, through interactions with cell surface receptors, and indirectly by binding to other matrix proteins such as fibronectin and altering their interaction with cells (6, 11). TN has been shown to promote cell migration (12, 13), inhibit focal adhesion formation (14), induce cell proliferation (15, 16), and, in some cases act as a cell survival factor (17). TN promotes angiogenesis (18) and also induces expression of genes that modify ECM, such as matrix metalloproteinases (19), which themselves have been implicated in promoting tumor growth and invasion. The potential role of TN in the development of breast cancer has been emphasized recently (20), and it has been suggested that this protein may be an appropriate target for tumor immunotherapy. TN, however, is not a single protein. It exists as multiple isoforms, and the precise structure of these isoforms determines the ultimate effect of the protein. The oligomer comprises a linear arrangement of domains, with a cysteine-rich NH₂ terminus termed the tenascin assembly domain, followed by a series of EGF-like repeats, 8–17 FNIII-like repeats and a fibrinogen-like domain at the COOH terminus (Fig. 1). The structure and size of TN varies as a result of alternative splicing of exons within the FNIII repeat domain (6). A number of biologically active sites have been mapped to the FNIII repeat domain including recognition sites for cell surface receptors such as integrins, cell adhesion molecules of the immunoglobulin superfamily, and annexin II (6), as well as sites susceptible to proteolytic cleavage by matrix metalloproteinase (21). Thus, inclusion or exclusion of different exons in this region can generate considerable functional diversity, and evidence from in vitro studies using recombinant proteins and proteolytic fragments of TN demonstrate that different isoforms differently affect adhesion, migration, and proliferation (22, 23).

Despite this, there are few studies examining TN isoform expression in malignancy, and most have investigated only the two most common isoforms, the “small” or fully truncated TN and the “large” unspliced molecule (ITN), containing all of the FNIII repeats. These studies indicate a switch in dominance from the small to large isoform in breast (24), oral (25), and colorectal (26) cancers. However, it is likely that more subtle changes in isoform expression occur that could have a profound effect on tumor cell behavior, and that these changes occur at an early stage in tumor progression. The aim of this study, therefore, was to analyze in detail TN isoform expression in benign and malignant breast tissue and to ask whether changes occur before tumor invasion and may therefore be implicated in promoting initial invasion.

MATERIALS AND METHODS

Cell Lines and Primary Cultures. All cell lines were obtained from American Type Culture Collection. MCF-7, T47D, MDA-MB 231, MDA-MB 468, and HBL-100 were maintained in DMEM plus 2 mM l-glutamine and 10% FBS; MCF-10A cells were maintained in 1:1 mixture of Ham’s F12 (Life Technologies, Inc.) and DMEM, 2 mM glutamine supplemented with 5%
Fig. 1. Schematic diagram of the structure of TN oligomer. TN comprises an NH2-terminal, cysteine-rich domain, 14 EGF-like repeats, a region of 8–17 FNIII repeats, and a COOH-terminal fibronectin (FG)-like sequence. Shaded boxes, exons that undergo splicing. AD1 and AD2, additional domains 1 and 2, respectively.

heat-inactivated horse serum, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 20 ng/ml EGF (all obtained from Sigma), and SK Mel 28 in MEMa plus 10% FBS. At 80% confluence, the medium was replaced with serum-free DMEM; the cells were incubated for 48 h and then harvested for mRNA extraction and RT-PCR. Primary cultures of normal breast myoepithelial cells were established from reduction mammary tissues after informed patient consent. Briefly, tissue was minced and incubated for 12–18 h in a digestion mixture of 200 units/ml collagenase type IA and 125 units/ml hyaluronidase in DMEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. After washing twice in cold DMEM + 10% FBS, a single sedimentation step of 30 min at 4 °C was carried out, and the supernatant was removed. For the isolation of myoepithelial cells, remaining organoids were washed and further digested in trypsin/EDTA (Sigma) with 10 mg/ml DNase I (Sigma) for a maximum of 10 min to generate a single cell suspension. Myoepithelial cells were then isolated using CALLA-labeled sheep antineu IgG1 magnetic beads (Dynal, Bromborough, United Kingdom) and plated in Specialized Media consisting of 1:1 Ham's F12:DMEM with 10% FBS, 5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml EGF, and 100 IU penicillin and streptomycin (all from Sigma). At subconfluence, the cells were transferred to serum-free DMEM for 48 h before extraction of mRNA.

**Tissue**. Breast tissue samples, obtained in accordance with current local ethical recommendations, were snap frozen in liquid nitrogen onto cork and stored in the vapor phase of a liquid nitrogen freezer. Parallel slices of tissue were fixed in 10% formal saline for 24 h at room temperature and routinely processed.

Analysis was performed on 15 normal/benign cases, 5 fibroadenomas, 13 cases of DCIS, and 35 carcinomas including 28 infiltrating ductal carcinomas. Breast tissue samples, obtained in accordance with current local ethical recommendations, were snap frozen in liquid nitrogen onto cork and stored in the vapor phase of a liquid nitrogen freezer. Parallel slices of tissue were fixed in 10% formal saline for 24 h at room temperature and routinely processed.

**RT-PCR**. mRNA was extracted and processed using oligo-dT-linked Dynabeads (Dynal), as described previously (29). To ensure approximately equivalent amounts of mRNA in reactions, the sections were scored as high, medium, or low cellularity, and 5, 10, or 15 × 7-μm sections were taken for mRNA extraction. The sections were immediately dropped into 100 μl of lysis/binding buffer [100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA (pH 8.0), 1% w/v SDS, and 5 mM DTT] and incubated with 50 μg/ml proteinase K (Boehringer Mannheim) for 1 h at 37 °C. The lysate was centrifuged for 30 s at 10,000 × g, and the supernatant was mixed with oligo-dT-linked Dynabeads (Dynal). The mRNA was allowed to anneal to the Dynabeads for 10 min at room temperature. mRNA-linked Dynabeads were washed twice in a buffer containing LiDS [10 mM Tris-HCl (pH 8), 0.15 M LiCl, 1 mM EDTA, and 0.1% LiDS; Dynal] and three times in the same buffer but without LiDS. Dynabeads were finally resuspended in diethyl pyrocarbonate water.

**Sequencing**. A direct method of sequencing was applied to isolated agarose gel electrophoresis-purified PCR products. In brief, PCR products were purified from 1% NuSieve agarose gel after electrophoresis. Forward strands were immobilized by the addition of Dynabeads M-280 Streptavidin (Dynal) and subsequently denatured with 0.1M NaOH. The biotinylated strand was isolated using the Dynal magnetic particle concentrator and then washed to remove the other strand. The resultant beads were then used as a template for sequencing using BigDye terminator cycle ready reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer). BigDye terminator reactions were run on an ABI Prism 377 DNA sequencer, and the resulting sequence profiles were analyzed using the Chromas software.

**Quantitation of Specific Isoforms**. To amplify individual isoforms, a number of PCR primers were designed across exon boundaries (Fig. 2b). When used with T15R primer, the 9–17 primer across the boundaries of exons 9 and 17 would amplify only the truncated isoform, the 9–16 primer across the boundaries of exons 9 and 16 would amplify only the exon 16-containing isoform, and the 14–16 primer across exons 14 and 16 would amplify only the isoform containing exons 14 plus 16. Optimization of these primer sets was undertaken on the appropriate gel-extracted single isoform at a range of annealing temperatures, and only when a single product of the expected size was obtained were they applied to primary tissues.

**Southern Blotting**. Southern analysis of RT-PCR products was performed using a modification of the method described by Southern (30). Oligonucleotide probes specific to each exon within the variable region (Table 1) were labeled with digoxigenin-11-dUTP in a reaction mixture containing reaction buffer (0.2 mM potassium cocodylate, 25 mM Tris-HCl, and 0.25 μg/ml BSA, pH 6.6), 5 mM CoCl2, 0.08 μM digoxigenin-11-dUTP, 0.4 mM dATP, 45 IU terminal deoxynucleotidyl transferase, and 1 μg of oligonucleotide at 37 °C for 15 min. The reaction was halted by addition of 0.5 mM EDTA.

After prehybridization in 5× SSC, 30 μg/ml denatured salmon sperm DNA, 0.1% N-laurylsarcosine NaCl, 0.2% SDS, 35% deionized formamide, and 2% w/v blocking reagent (Boehringer Mannheim) in wash buffer (1 mM Tris, pH 7.5) for 1 h at 37 °C, the membranes were probed with single oligonucleotides at 5 ng/ml in hybridization buffer for 18 h at 37 °C. Three times 15-min posthybridization washes in 2× SSC, 0.1% SDS, 35% formamide were carried out at 37 °C with a final 10-min wash in wash buffer with 3% Tween 20.

**Immunolological Detection**. Immunolological detection used anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim; 1:10,000), followed by chemiluminescent detection (Boehringer Mannheim).

**Table 1**. Position of primers across exon boundaries for isoform-specific amplification. This illustrates the position and combination of PCR primers designed for the selective amplification of truncated TN, TN16, and TN14/16, respectively.
The table details the nomenclature of the primers used and their sequences and positions within the gene according to base pair number and exon position. The position according to FN repeat, a terminology used by some workers, is also given.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Exon</th>
<th>Position (bp)</th>
<th>Region</th>
<th>FN repeat</th>
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<tbody>
<tr>
<td>EGFF</td>
<td>5’-TTC TGC TGA CTG TCA CAA TC-3’</td>
<td>2</td>
<td>1266–1285</td>
<td>EGF</td>
<td>NA</td>
</tr>
<tr>
<td>EGFR</td>
<td>5’-TGC TCA CAT ACA CAT TGG GC-3’</td>
<td>2</td>
<td>1508–1489</td>
<td>EGF</td>
<td>NA</td>
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<tr>
<td>T8F</td>
<td>5’-CAA TCC AGC GAC CAT CAA GC-3’</td>
<td>8</td>
<td>3057–3076</td>
<td>com.</td>
<td>4</td>
</tr>
<tr>
<td>T11F</td>
<td>5’-CAA TTG GGG GGT GGT GCT GGG-3’</td>
<td>11</td>
<td>3633–3652</td>
<td>var.</td>
<td>7 (A2)</td>
</tr>
<tr>
<td>T11P</td>
<td>5’-AEG ACC TCT CCC AAA TTA TG-3’</td>
<td>11</td>
<td>3650–3631</td>
<td>var.</td>
<td>7 (A2)</td>
</tr>
<tr>
<td>T12P</td>
<td>5’-GCC TGG GAT TCC CAT GGA AC-3’</td>
<td>12</td>
<td>4080–4061</td>
<td>var.</td>
<td>8 (A3)</td>
</tr>
<tr>
<td>T13P</td>
<td>5’-TCT CCC AGC TGT GGG AGA AC-3’</td>
<td>13</td>
<td>4190–4171</td>
<td>var.</td>
<td>9 (A4)</td>
</tr>
<tr>
<td>T14P</td>
<td>5’-TCT GGT GAA CGA CAA ACT GC-3’</td>
<td>14</td>
<td>4595–4613</td>
<td>var.</td>
<td>10 (B)</td>
</tr>
<tr>
<td>T14P</td>
<td>5’-GTT CTG TCA GCA CCA CAG AT-3’</td>
<td>14</td>
<td>4610–4591</td>
<td>var.</td>
<td>10 (B)</td>
</tr>
<tr>
<td>AD1P</td>
<td>5’-GCA GTG AGT GAG CCT TC-3’</td>
<td>AD1</td>
<td>130–111</td>
<td>var.</td>
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</tr>
<tr>
<td>AD2P</td>
<td>5’-ATC TCG TGG GAA CTA GCT C-3’</td>
<td>AD2</td>
<td>61–80</td>
<td>var.</td>
<td>AD2</td>
</tr>
<tr>
<td>AD3P</td>
<td>5’-GAC AGA CCT TAA CCT GAA CAC C-3’</td>
<td>AD3</td>
<td>175–159</td>
<td>var.</td>
<td>AD2</td>
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<tr>
<td>T15P</td>
<td>5’-TTA GGT TTT TCA GAA GGG GC-3’</td>
<td>15</td>
<td>197–179</td>
<td>var.</td>
<td>AD2</td>
</tr>
<tr>
<td>T16P</td>
<td>5’-GTT GTC AAC TTC CGG TTC GG-3’</td>
<td>16</td>
<td>5290–5271</td>
<td>var.</td>
<td>12 (D)</td>
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<tr>
<td>T18R</td>
<td>5’-CCT GCA CAG TTA CCA TCG AG-3’</td>
<td>18</td>
<td>5410–5391</td>
<td>con.</td>
<td>13</td>
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<tr>
<td>T25F</td>
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<td>6323–6342</td>
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<tr>
<td>T26P</td>
<td>5’-TGA TGG CTG ATG TTG CTC C-3’</td>
<td>26</td>
<td>6541–6522</td>
<td>Fib.</td>
<td>NA</td>
</tr>
<tr>
<td>T27R</td>
<td>5’-CAG TGG TAC CAG TTT TCA ACG C-3’</td>
<td>27</td>
<td>6656–6637</td>
<td>Fib.</td>
<td>NA</td>
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<tr>
<td>9/17F</td>
<td>5’-CA TCC ACT GCC ATG GCC GC-3’</td>
<td>9/17F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>9/18F</td>
<td>5’-C AC CTC TGA AGC CCA AC-3’</td>
<td>9/18F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>9/14F</td>
<td>5’-G CAT CTC CCA CGG CCA AGA A-3’</td>
<td>9/14F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>14/16R</td>
<td>5’-CTC GCC TCC TTC TGT GGC GGG-3’</td>
<td>14/16R</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</table>

* F, forward primer; R, reverse primer; P, oligonucleotide probe; NA, not applicable; /, exon boundary; con., conserved exon; var., variable; Fib., fibrinogen-like domain

**Immunohistochemistry.** Immunohistochemical detection of TN was performed on cryosections taken from the same tissue blocks used for RT-PCR analysis using two mouse monoclonal antibodies, the first against an epitope in the conserved EGF-like repeat region of TN (BC-24; Sigma, United Kingdom) and the second specific to a domain within exon 14 of the variable region of TN (BC-25; Chemicon; Ref. 31). Sections were incubated with the primary antibody (BC-24 at 1:7500 and BC-25 at 1:1000) for 18 h at 4°C and localized using a standard avidin-biotin complex (DAKO) technique. For each case, a negative control with omission of the primary antibody was included. In each case, the distribution of staining was recorded by two independent observers.

In Situ Hybridization. To localize TN mRNA expression, single-stranded antisense probes for nonisotopic in situ hybridization were synthesized using asymmetric PCR (32, 33). Briefly, a biotinylated reverse/antisense primer was used to generate an antisense probe from the sense/coding strand of the PCR product. Digoxigenin-11-dUTP was used as a replacement for a proportion of the nucleotide dTTP to produce a labeled probe. A total TN probe was prepared using products generated from PCR with EGF primers, fully truncated TN using products from 8/18 PCR, and a probe to exons 14–16 using primers between 14/18.

Asymmetric PCR was performed with 1 μl of a RT-PCR product as a template for the reaction amplified previously with a biotinylated forward primer. The reaction included 70 μM digoxigenin-11-dUTP (Roche Molecular Biochemicals, Lewes, United Kingdom) and 100 pmol reverse primer in reaction buffer as above. One IU Taq polymerase (Promega) was added after an initial denaturation to 98°C, and amplification was carried out for 20 cycles. Double-stranded contaminant was removed by incubation with Strepavidin-Dynabeads (Dynal) to give single-stranded digoxigenin-labeled probe. A positive control probe to β-actin was synthesized in the same manner, a negative sense probe was prepared using a forward primer in the asymmetric reaction, and template was prepared from RT-PCR reaction performed using a biotinylated reverse primer.

In situ hybridization was carried out on dewaxed rehydrated tissue sections. Pretreatment with proteinase K at 2–10 μg/ml in 50 mM Tris-HCl (pH 7.6) at 37°C was optimized for each tissue section. Sections were incubated in prehybridization solution [0.6 M NaCl, 50 mM Tris-HCl, 5 mM EDTA, 0.1% sodium P04, 0.2% polyvinylpyrrolidone (M40,000), 0.2% Ficoll (M400,000), 10% dextran sulfate, 0.15 mg/ml salmon sperm DNA, and 50% formamide] for 1 h at 37°C, followed by hybridization with the digoxigenin-labeled probe in the same solution for 18 h at 37°C. Slides were then washed twice in 2× SSC and 50% formamide, incubated with 1:25 unit/ml alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals), and washed in TBS-c containing 150 mM sodium chloride (pH 7.6). Signal was detected by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) containing 1 mM levamisole (Sigma). The optimum probe concentration was titrated to eliminate background staining while retaining good signal strength. Negative controls were carried out using sense probe to tenasin, RNase pretreatment of the tissue sections, and by omission of the probe in the hybridization protocol. Unbilled cord was used as a positive control tissue.

**Invasion Assays.** The invasive potential of the breast cell lines was measured using a modification of the Boyden chamber invasion assay used by Albini et al. (34) as described previously (35). Each assay was performed a minimum of three times, and a MI was calculated.

**RESULTS**

Detection of TN Isoform Expression in SK Mel-28 Cells

The malignant melanoma cell line SK Mel-28 has been described previously as expressing multiple TN isoforms (36), and cDNA from these cells was therefore used to confirm that specific isoform expression could be detected. PCR was performed using multiple combinations of primers including 8/18, 8/11, 8/12, 8/13, 8/14, 8/AD1, 8/15 8/16, and 14/18. Primer sets located within the alternatively spliced domain including 11/AD1 and 14/AD1 were also used. The products were then subjected to Southern blotting and hybridized with exon-specific oligonucleotide probes. Both high and low molecular weight species were identified in keeping with previous reports, including AD1-containing species.

**RT-PCR and Southern Blotting Analysis of TN Isoform Expression in Primary Breast Tissue**

Assessment of Tissue mRNA Integrity and Comparison of Sample Cellularity. To assess integrity of mRNA within the samples, because degradation takes place from the 5’ to the 3’ end of the transcript, PCR was performed using primer sets for the EGF-like repeat domain at the 5’ end of the gene and for the fibrinogen-like domain at the 3’ end of the gene. A weaker intensity signal from the 5’ primer set compared with the 3’ set indicates a degree of mRNA degradation within the sample, and such cases were excluded from further analysis.

PCR was also performed with primers to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, and the relative intensity of bands obtained was compared between different samples. The amount of template added to each reaction was adjusted on the basis
of the glyceraldhe-3-phosphate dehydrogenase result to ensure approximately equivalent amounts of cDNA in each reaction.

**Analysis of TN Isoform Expression with the 8/18 Primer Cassette.** Using 8/18 primers, three patterns of isoform expression were identified in the breast samples (Fig. 3). The first consisted of a single band of ~442 bp, indicating the fully truncated form of TN (tTN). The second pattern comprised two bands, the 442-bp band plus a band of 715 bp, indicating inclusion of one additional exon. The third pattern consisted of tTN, a plus-one exon species and a band of 1050 bp, indicating inclusion of two additional exons. Southern blotting followed by hybridization with exon-specific probes confirmed the 442-bp band to be the tTN and demonstrated the two additional bands as containing exon 16 (TN16) and exons 14 plus 16 (TN14/16), respectively (Fig. 3). The identity of these isoforms was also confirmed by sequencing. The shadow bands detected beneath each major isoform band were found on cloning and sequencing to contain a dual isoform population (data not presented) and are thought to represent hybridization between the isoforms present in the PCR mixture.

Differences in isoform expression were identified between the tissue samples. Seventy % (14 of 20) of normal/benign cases expressed only tTN compared with 7% (3 of 35) of invasive carcinomas. In contrast, 75% (27 of 35) invasive breast carcinomas were found to express both TN16 and TN14/16, and 14% (5 of 35) expressed TN16 in addition to tTN, whereas only 1 benign sample, a cellular fibroadenoma, expressed TN14/16 (Fig. 4). In all cases, TN 16 was expressed in association with TN14/16. The expression of either TN16 or TN14/16 plus TN14/16 was significantly related to the malignant (i.e., DCIS and invasive) phenotype (P < 0.0001), whereas expression of TN14/16 was significantly associated with the invasive phenotype (P < 0.0001). For the 13 cases of DCIS, 6 expressed all three isoforms, 4 showed tTN plus TN16, and 3 expressed tTN only (Fig. 4). Two of the three cases exhibiting tTN only were of low/intermediate nuclear grade, whereas the remainder of the cases were high grade in type.

**Semiquantitative Analysis of Specific Isoforms in Relation to Total TN.** PCR primer sets were designed to amplify tTN, TN16, and TN14/16 isoforms specifically to assess the relative abundance of these isoforms in relation to total TN production. PCR was performed initially using single isoforms extracted from agarose gels as templates for primer cassettes 9–17/18, specific for tTN, 9–16/18 specific for TN16, and 9–14/16–18 specific for TN14/16. However, primer cassette 9–17/18 gave a band with all three isoforms under all PCR conditions, whereas the 9–16/18 primer set amplified both TN16 and TN14/16 species. Only the 9–14/16–18 primer set gave specific amplification of the TN14/16 isoform. This primer set was therefore used to demonstrate the presence of the TN14/16 isoform and to compare relative amounts of this isoform with total TN.

A nested PCR procedure was used using products from 8/18 PCR with cycle sampling at 30, 35, and 40 cycles, followed by second round PCR with 9–14/14–16 primers for 25 cycles. This was compared with levels of total TN using fibrinogen-repeat primers at 30 and 35 cycles. A clear band for total TN was generated in each sample at 35 cycles; however, differences in band intensity at 30 and 35 cycles indicated differences in abundance of mRNA for TN in the different samples (Fig. 5). With the 9–14/14–16 primer set, 86% (24 of 28) invasive carcinoma samples exhibited a band, indicating expression of the TN14/16 isoform, compared with 73% (8 of 11) cases of DCIS and 50% (7 of 14) benign samples. The detection of the TN14/16 isoform using this approach was significantly related to the malignant phenotype (P < 0.033), and the relative intensity of bands achieved for TN14/16 and total TN suggests that TN14/16 was not being detected simply because more total TN was present in the tissue. Five fibroadenomas were also analyzed using this primer set, and 4 exhibited a band for the TN14/16 isoform. When fibroadenomas are included with the benign sample group, the significant relationship of TN14/16 detection with the malignant phenotype was lost (P < 0.062).

**Analysis of TN Isoform Expression with 11/16 Primer Set.** A series of 10 samples were further analyzed using the primer cassette 11/16. Four patterns of expression were detected: (a) no signal, indicating that no isoforms containing these exons are present; (b) a band of 1025 bp, indicating isoforms containing 3 exons between exons 11 and 16; (c) bands of 1025 and 812 bp, indicating species containing 3 and 2 exons, respectively, between exons 11 and 16; and (d) three bands including the 1025- and 812-bp species described, and a third band of 536 bp, indicating addition of 1 exon between exons 11 and 35.

**Fig. 4.** Relationship between tissue type and pattern of tenasin isoform expression. The graph shows the number of cases of each histological type expressing tTN alone, tTN plus TN16 or tTN, TN16 and TN14/16. A highly significant relationship is demonstrated between the expression of either TN16 or TN16 plus TN14/16 and the malignant phenotype (P < 0.001) and between the expression of TN14/16 and the invasive phenotype (P < 0.001).
11 and 16 (Fig. 6). All but one malignant sample expressed the largest isoform with three additional exons between exons 11 and 16, whereas only 1 benign sample showed this pattern of isoform expression. The intermediate-sized isoforms containing one or two additional exons were expressed only in invasive carcinoma samples, with no detection in the benign or DCIS cases. Unfortunately because of limiting amounts of mRNA from appropriate samples, it was not possible to characterize these exons by Southern blotting.

**RT-PCR for AD1-containing Isoforms.** In addition to PCR with 8/18 primers flanking the entire alternatively spliced domain, PCR was performed with the 8/AD1 primer set. No product was obtained with the 8/AD1 primers, indicating that no AD1-containing isoforms were expressed in benign, DCIS, or malignant breast tissue.

**Expression and Distribution of TN Proteins in Primary Breast Tissue**

Immunohistochemistry was performed on all samples analyzed by RT-PCR using BC-24 that recognizes all TN isoforms, thereby indicating distribution of all TN proteins and αIIIb specific to a domain within exon 14 of TN. In the normal/benign cases, strong intensity staining for total TN was detected in the basement membrane zone of ducts and acini as well as in the walls of blood vessels (Fig. 7a). There was no significant staining localized to the stromal compartment of these cases. In contrast, all invasive carcinomas demonstrated extensive diffuse stromal staining around tumor groups (Fig. 7b). In DCIS, a variable pattern of reactivity was observed; all cases revealed staining in the basement membrane zone; however, around some ducts, this was discontinuous. In addition, 9 of 13 cases exhibited laminations of staining in the stroma surrounding the abnormal duct (Fig. 7c).

The majority of normal/benign ducts and lobules showed no reactivity with the antibody to exon 14 (Fig. 7d). In contrast, the pattern of staining in DCIS was similar to that seen with the total TN antibody, with the same 9 of 13 cases exhibiting periductal stromal reactivity (Fig. 7e), and intense reactivity was observed in the peritumoral stroma of all of the invasive carcinomas (Fig. 7f). However, seven cases of normal breast revealed intense basement membrane zone staining in one or more acini within a lobule or around a duct (Fig. 7, g and h). Interestingly, in many of the normal cases, reactivity for the exon 14 antibody was seen in the wall of large and small blood vessels included in the tissue section (Fig. 7i). All of the fibroadenomas also displayed diffuse stromal staining with this antibody.

**Localization of TN mRNA in Breast Tissue**

*In situ* hybridization was performed on 18 cases using single-stranded asymmetric probes to β-actin, tTN, total TN, and species containing exons 14–17. A strong signal for β-actin was demonstrated in all cells of each case, indicating good mRNA integrity. In normal and benign breast tissues, no signal was generated with any of the TN probes (Fig. 7, j and m), whereas in the invasive carcinomas, strong signal was generated for total TN, tTN, and TN14–16, in each case being localized to the peritumoral stroma (Fig. 7, l and o). No convincing signal was localized to the tumor cell population. In the DCIS cases, signal was exhibited for all three TN probes in myoepithelial cells around affected ducts and in the periductal fibroblasts (Fig. 7, k and n). Tissue incubated with sense probes or in the absence of probe exhibited no signal, confirming the specificity of the reaction.

**Relationship between TN Expression and Invasive Activity by Breast Cell Lines**

Invasion assays were performed on four breast cancer cell lines (Fig. 8a). These showed that the MDA-MB 231 cells were the most highly invasive with a MII of 61.5% (SD, 4.66). The MDA-MB 468 cells were also highly invasive with a MII of 51.8% (SD, 2.46), whereas the MCF-7 and T47D cells were markedly less invasive in this assay with MII of 31.0% (SD, 1.50) and 12.0% (SD, 1.96), respectively.

RT-PCR for total TN demonstrated that TN was produced by MDA-MB 231 and MDA-MB 468 breast cancer cells but not by MCF-7 or T47D cells. Tenascin was also expressed by myoepithelial cells and the two nontumorigenic cell lines HBL-100 and MCF-10A (Fig. 8b).

**DISCUSSION**

TN is a large glycoprotein that is highly expressed in many solid tumors (26, 37). Multiple isoforms may be generated through alternative splicing of exons within the FNIII repeat region, and these isoforms have been shown to exert different effects on aspects of cell behavior such as proliferation and migration (12, 15, 16). Developmental studies indicate strict temporal and spatial control of isoform expression (38), implying a structure-function relationship; however, few studies have addressed whether distinct patterns of TN isoform expression occur with the development and progression of malignancy. Using a combined RT-PCR and Southern blotting approach, this study has identified consistent differences in the pattern of isoform expression between benign, preinvasive, and invasive breast.

![Figure 6](image-url)
lesions. Using the 8/18 primer cassette that spans the entire alternatively spliced region, the fully truncated form of TN (tTN) was identified in all samples; however, in a proportion of cases, two further isoforms were identified, one containing exon 16 (TN16) and one containing exons 14 plus 16 (TN14/16). The expression of both TN16 and TN14/16 was significantly associated with the malignant phenotype, whereas expression of TN14/16 was related to the invasive phenotype. An important issue to be considered in the interpretation of these results was that detection of these higher molecular weight fragments in malignant samples represented a true qualitative change in expression and was not simply a reflection of higher levels of TN. In some samples, the bands for TN16 and TN14/16 were of greater intensity than tTN, suggesting a switch in isoform predominance rather than an increase in all TN isoforms. To address this more directly, primers were designed across exon boundaries to specifically amplify tTN, TN16, and TN14/16 in separate reactions. Using the

Fig. 7. Expression and distribution of tenascin protein and mRNA in breast tissue. Using an antibody that recognizes all TN isoforms (Total TN), strong linear staining is seen in the basement membrane region of normal ducts and acini (a), in contrast with the diffuse stromal reactivity around invasive carcinomas (c). Stromal reactivity is also present around ducts containing DCIS (b). With the antibody specific to exon 14 of TN (TN14), most normal breast ducts and acini did not exhibit staining (d), with only occasional ducts and acini in 7 cases revealing basement membrane reactivity (g and h). Blood vessels in normal breast displayed consistent reactivity with Ab14 antibody (i). A similar pattern of staining as that seen for total TN was present in DCIS (e) and invasive carcinomas (f), indicating the presence of exon 14-containing higher molecular weight species in tumor-associated stroma. No signal was generated in normal breast tissue using probes to total TN or tTN (j and m). In contrast, strong signals were generated in the fibroblasts surrounding invasive tumor groups (l and o). In DCIS, a signal with each probe is evident in residual myoepithelial cells surrounding affected ducts (arrow, k and n) and in periductal fibroblasts (arrowhead, k and n). An identical pattern of reactivity was seen with probes to TN14–17 (not illustrated).
purified isoform as template, only the 9–14/14–16 primer set could be optimized to amplify a single isoform, probably because of the high degree of homology between the different exons, particularly at the exon borders. When the 9–14/14–16 primer set was applied to the tissue samples, a product was obtained in 86% of invasive carcinomas and 73% of DCIS cases, confirming expression of the TN14/16 isoform in the majority of malignant samples. The relative intensity of total TN expression, assessed using primers to the fibrinogen-repeats region, compared with the relative intensities of bands obtained for TN14/16, indicated that strong expression of TN14/16 was not invariably related to higher levels of expression of total TN, further supporting a qualitative as well as quantitative change in TN expression. Interestingly, a higher proportion of benign cases exhibited a band for TN14/16 using the specific isoform primers compared with the 8/18 primer cassette. This is likely to relate to the enhanced sensitivity of the nested PCR approach used with the specific isoform primers and indicates that small amounts of TN14/16 are present in some benign tissues. Evidence for the presence of higher molecular weight species of TN in normal/benign breast was demonstrated by immunohistochemistry, which revealed staining with the exon 14 antibody in occasional glands but more abundantly and consistently in the wall of blood vessels. Thus, it is possible that the product for TN14/16 on RT-PCR generated from some benign cases relates to TN production in smooth muscle or endothelial cells. However, even using this highly sensitive technique, 50% of nonfibroadenoma benign cases did not yield a product for TN14/16, demonstrating that this isoform is certainly much more abundant in malignant tissue.

Studies that have used similar approaches have also identified specific changes in TN isoform expression associated with malignancy, although these differences appear to be tissue type specific. In renal cancer, the fully truncated isoform is predominant (39); however, the TN14/16 isoform has been related to the malignant phenotype in previous studies in malignant ovarian tumors (40), and in some tumor cell lines (36), although it is not a major isoform in normal tissues such as fetal membranes (41), raising the suggestion that this isoform may have functional significance in terms of tumor progression. Of particular interest is the finding of a similar altered isoform profile in a subset of preinvasive lesions with 46% of cases exhibiting TN16 and TN14/16, 31% expressing TN16, and 23% of cases producing iTN only. To address whether such altered isoform expression can predict for tumor progression would require a larger study with comparison to grade, recurrence, and presence or absence of micro-invasion.

Other changes in isoform expression were also identified using different combinations of primer cassettes. Although analysis was performed on a limited number of cases, differences between benign and malignant tissue were detected using the 11/16 primer set, with the majority of cases expressing larger isoforms containing three additional exons between 11 and 16, whereas only DCIS and invasive carcinomas exhibited isoforms of intermediate size with one or two additional exons. The detection of these larger isoforms appears to contradict the results obtained using the 8/18 primer cassette, which suggest that only isoforms with 16 or 14 plus 16 exons are present; however, this is likely to result from amplification of low abundance higher molecular weight species using primers internal to the FNIII-repeat domain. This is in keeping with previous studies that indicate the presence of low levels of high molecular weight species in normal and benign breast tissue (24). Because of the limiting amounts of mRNA, Southern blotting was not performed on the products obtained with this primer set; however, the consistency observed in splicing patterns suggests that these bands correspond to a plus 12, 15, and 14 isoform, with the plus-two-exon isoform containing either 12 or 15 and the plus-one-exon isoform lacking both 12 and 15. Thus, the smallest isoform would correspond to TN14/16. The detection of a similar TN isoform profile, with increased abundance of intermediate-sized isoforms in malignancy, has also been reported in ovarian tumors (40).

Genomic sequencing has identified two novel repeats, initially in chicken and later in human tenasin, termed “additional” repeats AD-1 and AD-2 (42, 43). AD-1 has been shown to be highly expressed at sites of tissue remodeling in embryonic tissues and also in tumor cell lines from breast, melanoma, and retinoblastoma (44), whereas AD-2 appears to be rarely expressed in human tissue (43). In the present study, there was no evidence of AD-1-containing isoforms in breast tissue.

The changes identified in TN isoform mRNA were associated with changes in the amount and distribution of the TN protein. In both DCIS and invasive tumors, stromal reactivity was detected, and the αIIIb antibody confirmed that this stromal expression included exon 14-containing species, which were largely undetectable in normal breast other than in relation to blood vessels. It has been suggested previously that expression of TN around DCIS correlates with grade...
(45) and is predictive of invasive behavior (8). This study demonstrates that as well as an increase in the amount of TN around DCIS, there is also a qualitative change, with the presence of higher molecular weight species, and it is possible that these may alter the microenvironment to promote invasion.

In situ hybridization demonstrated that total TN, tTN, and isoforms containing exons 14–18 are synthesized in the stromal compartment of invasive carcinomas and in residual myoepithelial cells and periductal fibroblasts of DCIS. No convincing signal was detected in the tumor cell population, which is in contrast to previous studies indicating that tumor cells may be the source of TN in breast carcinomas (46) and that this relates to a more aggressive phenotype (47). The lack of signal in tumor cells in this study most likely relates to selection bias in the cases studied, and a wider study is indicated not only to confirm the expression of TN by tumor cells but also to address whether tumor cells synthesize a different profile of TN isoforms compared with fibroblasts, which may influence tumor cell behavior. RT-PCR analysis of a series of breast cancer cell lines, however, supports the concept that tumor cells may produce TN. This demonstrated that both of the highly invasive tumor cell lines synthesized TN in contrast to the less aggressive cell lines, which showed no evidence of TN expression. Interestingly, two nonmucinogenic cell lines, HBL-100 and MCF-10A, and primary myoepithelial cells were also found to produce TN. Both HBL-100 and MCF-10A display features of a myoepithelial phenotype,4 and all of the cell lines that produce TN express the intermediate filament vimentin. This suggests that epithelial cell expression of TN is seen predominantly in cells with mesenchymal characteristics, either as part of their normal phenotype, as in myoepithelial cells, or as part of the epithelial-mesenchymal transition (48). No signal for TN mRNA was identified in normal breast tissue; however, the localization of TN protein to the basement membrane and the detection of TN expression in separated myoepithelial cell populations suggests that myoepithelial cells are the most likely source for TN in the normal breast, but that the rate of synthesis of TN in normal breast is below the threshold of detection.

In conclusion, this study has identified consistent and significant changes in TN isoform expression in malignant compared with normal/benign breast, associated with a change in the distribution of TN protein from the basement membrane zone to the peritumoral stroma and also in protein composition, with detection of exon 14-containing molecules. The presence of altered isoform expression in a subset of DCIS is of particular interest because this suggests there is remodeling of the stromal microenvironment before tumor invasion. Additional studies are now indicated to establish the functional and clinical significance of these TN isoforms and to identify their potential as predictive markers for tumor invasion. Clinical trials have already been carried out in patients with glioma using 131I-labeled antibodies to TN (49). Identification of specific isoforms that are involved in breast tumor progression would provide a powerful therapeutic target.

REFERENCES

Changes in Tenascin-C Isoform Expression in Invasive and Preinvasive Breast Disease


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