Isolation and characterization of human fibroblast tenascin. An extracellular matrix glycoprotein of interest for developmental studies

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Abstract We have developed a biochemical method for purifying human tenascin from cultured fibroblasts or the culture medium. The method is a series of biochemical procedures including gel filtration, gelatin gel affinity chromatography and ion-exchange high performance liquid chromatography. The final preparation was identified as tenascin from its immunological cross-reactivity to antibody against chicken tenascin, strong hemagglutination activity which has been reported to be one of the biological functions of chicken tenascin, and from the electron microscopic study demonstrating a six-armed structure. Gel chromatography showed that intact human tenascin has an apparent molecular weight of over one million. Analysis of the purified tenascin with SDS-PAGE under reducing conditions demonstrated that tenascin consists of two kinds of subunits (250K and 190K). We established rat x mouse heterohybridoma cell lines which produce tenascin-specific antibodies. One monoclonal antibody (RCB1) was selected for immunohistochemical study and partially characterized. RCB1 bound native tenascin but not reduced and alkylated tenascin. Immunohistochemistry of normal and neoplastic tissues demonstrated that RCB1 bound the connective tissues surrounding the cancer nests and various normal tissues including interstitium of renal distal tubule, periosteum, endostea, smooth muscles of digestive tract and media of arteries and arterioles.

Key Words: extracellular matrix, tenascin, fibronectin, fibroblast, human cancer

Introduction

Tenascin (TN), formerly known as “myotendinous antigen”, is an extracellular matrix (ECM) glycoprotein with a unique molecular structure. In common with the discoveries of other novel biological molecules, the original isolation of TN was serendipitous. A panel of mouse monoclonal antibodies was prepared against type V collagen from chicken, and one of these was unexpectedly reactive with myotendinous tissue by immunohistochemical staining (Chiquet and Fambrough, 1984; Chiquet-Ehrismann et al., 1986). This antibody, designated M1, was then used for immunoaffinity-purification of the protein from extracts of cultured fibroblasts. Subsequently, rotary shadowing electron microscopy revealed that the TN molecule is a six-armed, oligomeric macromolecule often referred to as “hexabrachion” in shape (Erickson and Inglesias, 1984; Erickson and Taylor, 1987; Chiquet-Ehrismann et al., 1988).

Although the biological functions of TN are presently unknown, a variety of experiments in vitro demonstrate that it is strongly hemagglutinating, promotes growth of cancer cells (Chiquet-Ehrismann et al., 1986) and inhibits cell attachment mediated by receptors for arginine-glycine-aspartic acid-serine (RGDS) (Chiquet-Ehrismann et al., 1988). TN is secreted in the mesenchymal stroma during organogenesis of mammary gland and tooth (Chiquet-Ehrismann et al., 1986; Inaguma et al., 1988), kidney (Aufderheide et al., 1987), and urogenital sinus (Takeda et al., 1988). It is also found along

Abbreviations used in this paper: TN, tenascin; FN, fibronectin; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PMSF, phenylmethylsulfonyl fluoride; CHAPS, (3-[3-cholamidopropyl]dimethylammonio)1-propanesulfonate); SDS-PAGE, sodium dodecyl sulfate polacrylamide gelelectrophoresis; PBS, phosphate buffered saline; BCA, bicinchoninic acid.

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migration pathways of embryonic neural crest cells (Theisieff et al., 1987). Mammary gland TN gradually disappears before adulthood in mice and rats, but it reappears in the stroma of cancerous tissue (Inaguma et al., 1988), suggesting that it may be an oncofetal protein. In addition, TN is present in extracts of cell surface proteins from human fetal fibroblasts and malignant tumors of mammary gland, esophagus, lung, liver, and urinary bladder (unpublished). It has been proposed that the extracellular deposition of TN may result from cell-cell interactions between mesenchyme and epithelium during fetal development and neoplastic transformation (Inaguma et al., 1988). In view of these observations, it is likely that TN plays a major role in intercellular communication during the cell proliferation and migration that accompanies morphogenesis and carcinogenesis.

Most biochemical and biological studies of TN historically have been conducted on cultured cells and tissues from embryonic and adult chickens. However, in order to better understand the role of TN in mammalian development and cancer, additional experimental model systems including those of the mouse, rat and human are required. Therefore, new methods of purification of TN from these species are needed. One strategy utilizes a rabbit polyclonal antibody prepared against chicken TN that cross-reacts with human TN (Mackie et al., 1987), but the yield of the procedure is limited by the efficiency and size of the immunoaffinity gel employed. An alternative approach takes advantage of the fact that TN is extracted along with fibronectin (FN) and cell surface proteins by high concentrations of urea (Chiquet-Ehrismann et al., 1986). FN, a major contaminant because of its affinity for TN, is then removed after binding to a gelatin-sepharose affinity column, and final purification of TN is achieved by high performance liquid ion exchange chromatography. We report here the details of this latter method for isolation of human TN, and we also describe the preparation of an anti-human TN monoclonal antibody for immuno-histochemical staining of surgical biopsies and autopsy tissues.

Results

Extractivity and subunit structure of human fibroblast TN

Fig. 1 illustrates the immunogenic profile of TN subunits extracted with 2M urea (a) and followed by 4M guanidinium chloride (b) on an immunoblot. These results indicate that TN subunits are composed of proteins with an apparent molecular weight of 250k and 190k, and TN molecule is efficiently extracted from cell layer with 2M urea (4M guanidinium treatment of 2M urea extract does not alter immunogenicity of TN when analyzed by Immunoblotting).

Purification of human fibroblast cellular TN

In terms of the biochemical nature of human TN, we found that a combination of gel filtration, gelatin affinity chromatography and DEAE-5PW procedure is successful for purification (Fig. 2). 2M urea extract (100 mg protein) was prepared from HUCF-P2 cells cultured in thirty roller bottles. The sample was applied to a Sepharose CL4B column. The protein content was determined by BCA assay (Fig. 3a) and immunogenicity was examined by ELISA (Fig. 3b). TN was eluted at the position of Kd=0.11, which indicates that TN has an apparent molecular weight of more than one million, while FN was eluted at Kd=0.24 with a shoulder at the same position where TN was eluted. The sample was collected from the fractions of Kd=0.11 (Fraction A). Protein content of this sample was 3 mg. Fraction A was then applied to gelatin Sepharose chromatography. The pass through fraction (3.3ml) was collected. Since no FN immunogenicity was detected in this fraction (result not shown), this step was successful for depleting FN completely. The yielded protein was 2.7 mg. The

2M Urea extract or 33.3% Ammonium sulfate precipitate from medium

↓

Sepharose CL4B (Fig. 3)

↓

Gelatin affinity chromatography

↓

DEAE-5PW chromatography (Figs. 4, 6)

↓

Collect Fraction II as purified TN

Fig. 2. Purification strategy scheme for human fibroblast TN.
fraction was then applied to DEAE-5PW ion-exchange HPLC column. Three major peaks termed “Fractions I, II and III” appeared on the elution pattern (Fig. 4a). Among these three Fraction II was identified as TN from the result of direct ELISA using TN antibody (Fig. 4b). Fraction III was a mixture of TN and proteoglycan (Fig. 4c). Coelution of TN and proteoglycan in Fraction III suggests that a part of TN was bound to proteoglycan under the elution condition that we employed. The identity of material eluted in Fraction II was tested further. An aliquot of each Fraction numbered 38 to 47 was analyzed by SDS-PAGE under the reducing condition. As shown in Fig. 5a, protein bands with apparent molecular weights of 250K and 190K were observed only in Fractions numbered 42 to 44. While under the non-reducing condition, this protein stayed at the top of the gel (Fig. 5c). These characteristics were similar to those of TN in the starting 2M urea extract, which were detected by TN antibody (see Fig. 1). In addition, Immunoblot analysis clearly indicates that these two bands are TN subunits (Fig. 5b). Fractions of numbers 42-44 were served for further analysis as purified TN. The yield of TN was 500 mg.

**Purification of TN from culture medium**

The ammonium sulfate precipitation gave 150 mg protein from 15 l of culture medium. The precipitate was dissolved in 20 ml of buffer A, and was applied to a Sepharose CL4B column. The subsequent steps, including gelatin Sepharose 4B gel chromatography and DEAE-5PW ion-exchange column chromatography, were the same as used for the purification of cellular TN. 450mg as protein was finally obtained. The elution profile and its immunoreactivity to anti-chicken TN and anti-chondroitin sulfate antibodies are illustrated in Fig. 6. The SDS-PAGE analysis of the final product and its immunoreactivity to TN antibody demonstrated the typical banding pattern of TN (Fig. 7). Comparing the result of biochemical analysis of medium TN with that of cellular TN, it is clear that both TNs have similar biochemical characteristics.

**Hemagglutinating activity of human TN**

The ability of TN to agglutinate formalinized sheep erythrocytes was tested. As Fig. 8 shows, TN purified from human fibroblasts had agglutinating activity at the minimal concentration 7.5µg/ml.

**Ultrastructure of human TN**

Rotary-shadowed sample of purified TN from cultured fibroblast was examined by electron microscopy. Several images of six-armed molecules with a central globule to which two pairs of three arms seem to be connected were observed (Fig. 9).
Fig. 5. SDS-PAGE analysis and Immunoblotting of Fraction II. (a) SDS-PAGE of the DEAE-5PW ion-exchange column fractions (numbers 40 to 46 shown in Fig. 4a) under reducing condition and (b) its Immunoblotting using chick TN polyclonal antibody. (c) SDS-PAGE of fraction 43 under non-reduced condition. Arrows indicate the positions of the molecular weight.

Establishment of monoclonal antibodies to human TN

Fifteen hybridoma cell lines that secreted immunoglobulins reactive to human TN were established. Among them, one monoclonal antibody (termed RCB1), whose subclass is IgG2a, was further characterized. When direct ELISA was performed using native TN, RCB1 was highly reactive against human TN (Fig. 10). The reaction was inhibited by the antibody with purified TN (data not shown). Staining profile of Immunoblot further confirmed that this antibody is specific for TN (Fig. 11). When ELISA was performed using reduced and alkylated TN as an antigen, no reaction was observed. This result suggests that RCB1 requires rather native state of antigen for its reaction and, further, it recognizes stereochemical information of the protein.

Immunohistochemistry of normal and neoplastic tissues

Normal and neoplastic tissues were examined in about 100 cases of autopsies. The positive tissues of bound RCB1 are summarized in Table 1. Among the tissues showing normal histology, interstitium of renal distal tubule (Fig. 12a), peristomeum and endostem of bone marrow (Fig. 12b) are the most common sites of positive staining. TN expression in the lamina propria (Fig. 12c), muscularis mucosae (Fig. 12d) and smooth muscle layers of digestive tract organs, subepidermal layer, the media of arteries and arterioles (Fig. 12e) and the periductal mesenchyme of bronchus and prostate are variable and differ according to cases and sites. Many other normal tissues including salivary gland, thyroid gland, pancreas, testis, placenta, uterus, bone, cartilage, cerebel- lum, blood cells and the epithelial components of all organs are negative. The connective tissues surrounding the cancer nests are usually stained positively (Fig. 12f).

Discussion

The major conclusions of this study may be summarized as follows: 1) TN was purified from extracts of cultured human umbilical fibroblasts and conditioned medium by gel filtration followed by gelatin-sepharose affinity column, anion exchange column, and high pressure liquid chromatography; 2) the protein was eluted with 0.25 M NaCl as a single peak from the anion exchange column; 3) it had an apparent molecular weight in excess of one million kDa, and it was composed of disulfide linked subunits with molecular weights of 250 kDa and 190 kDa; 4) the protein was immunoreactive with anti-chicken TN antibodies but not anti-human FN or proteoglycan antibodies by ELISA; 5) it had a high hemagglutinating activity; 6) a six-armed oligomeric macromolecular structure was revealed after rotary shadowing electron microscopy. Taken together, the findings provide strong evidence that the purified protein is indeed human TN.

Several of these points are worthy of elaboration. First, the six-armed oligomeric structure of human TN is identical to that reported previously for chicken TN (Erickson and Taylor, 1987; Chiquet-Ehrismann et al., 1988), the human glioma mesenchymal extracellular matrix (GMEM) protein (Bourdon et al., 1983), and the “hexabrachion” protein from human and chicken fibroblast cultures (Erickson and Inglesias, 1984). Second, SDS-PAGE electrophoresis of human fibroblast TN under reducing conditions demonstrated two protein bands at 250 kDa and 190 kDa, in contrast to the 220 kDa and 200 kDa bands that are characteristic of chicken TN. Human TN subunits extracted from other sources including normal and neoplastic tissues have apparent molecular weights that are the same as fibroblast TN. Post-translational modifications such as glycosylation of the TN subunits may be one possible explanation for these differences in electrophoretic mobilities. Comparison of the genomic nucleotide sequences of human and chicken TN DNA also may offer clues for understanding this unexpected result. With regard to the low molecular weight bands visible in the SDS-PAGE gel shown in Fig. 1 and the immunoblot in Fig. 7, pulse-chase analyses of TN synthesis in cell cultures suggest that they are mostly likely proteolytic degradation products. Third, like chicken TN, human TN has high hemagglutinating activity, and this property may be one of
Fig. 6. DEAE-5PW ion-exchange chromatography of Fraction A from conditioned medium after gelatin Sepharose 4B chromatography. Elution condition was same as in Fig. 4 and monitored at 260 nm absorbance (a). Distribution of immunoreactivity of TN (b) and chondroitin sulfate (c).

its biological functions (Chiquet-Ehrismann et al., 1986). Lastly, immunohistochemical staining with a monoclonal antibody prepared against human TN demonstrates reactivity of mesenchyme from a variety of human cancer tissues. Positive immunostaining also is apparent in the extracellular matrix of normal tissues including smooth muscle, blood vessels, liver sinusoids, and kidney mesenchyme (manuscript in preparation).

In conclusion, the problem of isolation and purification of human TN from cultured cells and tissues lies in its binding affinity for other ECM proteins including FN and proteoglycans (Chiquet-Ehrismann et al., 1988; Friedlander et al., 1988). This property may be more than just a troublesome experimental hurdle for biochemists. Local adhesive properties of the ECM substrate in relation to the cell surface, including the ability to bind growth factors, may form the molecular basis of cell-cell interactions required for embryonic morphogenetic movements, organogenesis, and migration of neoplastic cells. Future studies no doubt will shed light on these complex, dynamic interactions.

Materials and Methods

Materials
Human umbilical cord fibroblasts HUCF-P2 and mouse myeloma cell line P3xAg8.653 were provided by RIKEN gene bank. DMEM, 1:1 mixture of DMEM and Ham'S F-12 culture medium, PMSF, CHAPS, anti-chondroitin sulfate antibody (clone CS56), RPMI 1640 culture medium containing hypoxanthine/aminopterin/thymidine, RPMI 1640 containing hypoxantine/thymidine, polyethylene glycol (molecular weight 3,800) and pristan were from Sigma; fetal calf serum (fcs) was from Grand Island Biological Co.; cell culture bottles and ELISA plates were from Beckton and Dickinson; biochemical grade urea and ammonium sulfate were from Wako Pure Chemical Co.; 1BCA protein assay reagents were from Pierce. Rabbit polyclonal antibody against chicken TN was kindly supplied by Dr. R. Chiquet, FMI Basel; biotinylated rabbit anti-mouse IgG+IgA+IgM antibody, biotinylated rabbit anti-rat IgG+IgA+IgM antibody, biotinylated goat anti-rabbit IgG+IgA+IgM antibody, peroxidase-conjugated avidin, rabbit anti-human FN antibody and formalinized sheep erythrocytes were from Cappel; premade SDS poly-
acrylamide gel plates (4%-20%) were from Dai-ichi Pure Chemicals; pre-stained molecular weight markers (high range) were from Bethesda Research Laboratories; Sepharose CL4B, and gelatin Sepharose 4B were from Pharmacia Japan; DEAE 5PW(1cmx5cm), was from Tosoh. Other chemicals for SDS-PAGE were from Bio-Rad Japan. Nitrocellulose membrane (Nitro plus) for Immunoblotting was from Micromembrane Inc. Freund's complete and incomplete adjuvants were from Difco; Wistar rats and BALB/c mice were purchased from Charles River Japan.

**Cell culture**

HUCF-P2 cells were used as a source for TN extraction. They were maintained in a 1:1 mixture of DMEM-Ham F12 containing 10% fcs, penicillin (100 units/ml) and streptomycine (100 μg/ml) at 37°C. Cells were plated on 850 cm² roller bottles at a density of 2x10⁶ cells/ml. After these cells became confluent, 150ml of the culture medium was removed from each bottle and the same volume of the fresh medium was added. Repeating the medium collection with 5 days interval for 3 months, the conditioned media were pooled for the use of TN purification by the method described below. The cells were also used for the source of TN.

**Crude extraction of TN from cell layer**

2M urea extract was prepared from the HUCF-P2 cells according to the method described by Yamada and Akiyama, (1984). Briefly, the cells were washed three times with serum free DMEM containing 2mM PMSF for 30 min at 37°C. 2M urea in DMEM (20ml/bottle) was added to culture bottles and extracted for 2h. The extracts were precipitated with ammonium sulfate at 70% saturation (472 mg/ml of ammonium sulfate). The precipitate was collected with centrifugation at 20,000g for 30 min at 4°C and was dissolved in 2M urea/0.15M NaCl/0.05M Tris HCl pH 7.4/ 2mM PMSF (buffer A) and stored at -20°C until further processing of this fraction. In some experiments, materials in cell layers were further extracted with 4M guanidinium chloride/Tris HCl pH7.4/2mM PMSF (20ml/bottle).

**Crude fraction of TN from conditioned media**

To prepare crude TN from conditioned media, 15 l of conditioned media was mixed with 7.5 l of saturated ammonium sulfate (preliminary experiment 33.3% ammonium sulfate was found to precipitate TN efficiently
TABLE 1
MONOCLONAL ANTIBODY RCB1 REACTIONS WITH HUMAN TISSUES

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of cases</th>
<th>No. of positive(%)</th>
<th>Positive area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td>24</td>
<td>6(25.0%)</td>
<td>glia</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Digestive organs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
<td>29</td>
<td>21(72.4%)</td>
<td>lamina propria, muscle layer</td>
</tr>
<tr>
<td>Stomach</td>
<td>38</td>
<td>28(73.6%)</td>
<td>muscularis mucosa, muscle layer, perineurium of plexus mesentericus</td>
</tr>
<tr>
<td>Intestine</td>
<td>39</td>
<td>31(79.5%)</td>
<td>lamina propria, muscularis mucosa, muscle layer, perineurium of plexus mesentericus</td>
</tr>
<tr>
<td>Colon</td>
<td>38</td>
<td>26(68.4%)</td>
<td>muscle layer, muscularis mucosa, perineurium of plexus mesentericus</td>
</tr>
<tr>
<td>Liver</td>
<td>42</td>
<td>9(21.4%)</td>
<td>sinusoid</td>
</tr>
<tr>
<td>Pancreas</td>
<td>42</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Gall bladder</td>
<td>35</td>
<td>13(37.1%)</td>
<td>muscle layer, lamina propria</td>
</tr>
<tr>
<td>Salivary</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hematopoetic organs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>26</td>
<td>26(100%)</td>
<td>periosteum, endostemm</td>
</tr>
<tr>
<td>Spleen</td>
<td>38</td>
<td>10(26.3%)</td>
<td>trabecula, cord</td>
</tr>
<tr>
<td>Lymph node</td>
<td>37</td>
<td>30(71.4%)</td>
<td>capsule, trabecula</td>
</tr>
<tr>
<td>Endocrine organs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>4</td>
<td>4(100%)</td>
<td>intercellular space</td>
</tr>
<tr>
<td>Thyroid</td>
<td>45</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>38</td>
<td>26(81.1%)</td>
<td>striatum of medulla</td>
</tr>
<tr>
<td>Ovary</td>
<td>21</td>
<td>1(4.8%)</td>
<td>corpus luteum</td>
</tr>
<tr>
<td>Testis</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Urogenital organs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>49</td>
<td>49(100%)</td>
<td>interstitium of distal tubule</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>28</td>
<td>18(63.1%)</td>
<td>smooth muscle</td>
</tr>
<tr>
<td>Carcass</td>
<td>20</td>
<td>14(70.0%)</td>
<td>lamina propria</td>
</tr>
<tr>
<td>Prostate</td>
<td>15</td>
<td>12(80.0%)</td>
<td>periadrenal connective tissue</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>48</td>
<td>30(62.5%)</td>
<td>lamina propria of bronchus, perichondrium alveolar wall</td>
</tr>
<tr>
<td>Skin</td>
<td>24</td>
<td>9(37.5%)</td>
<td>subepithelial layer</td>
</tr>
<tr>
<td>Placenta</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>42</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Neoplastic</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary cancer</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>gastric cancer</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Colon cancer</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hepatoma</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pharyngeal cancer</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Giroma</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Uterine cancer</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

(more than 90%). The mixture was incubated at 4°C for at least 1h. The precipitate was collected by centrifugation at 10,000g for 30 min at 4°C and resuspended in a small volume of buffer A. The sample was stored at -20°C until use.

Gel filtration
Sephrose CL4B chromatography: Crude TN fraction from either cells or conditioned media was applied to Sephrose CL4B column (2.5 x 110 cm) which had been equilibrated with 2M urea/0.15 M NaCl/0.05 M Tris-HCl pH 7.4/2 mM PMSF/0.2% (v/v) CHAPS (buffer B). Concentration of protein was measured by BCA method (Smith et al., 1985). The elution was done at a flow rate of 20mL/h at 4°C with monitoring at 280 nm. The column was calibrated with following molecular weight marker FN, IgG and BSA. Fractions of about 6.5 mL were collected, and the immunoreactivity of each fraction was tested by direct ELISA.

Gelatin affinity gel chromatography
To remove FN from TN-enriched fraction (referred to as Fraction A) (see Fig. 3b) gelatin affinity chromatography was done by the method described by Chiquet-Ehrismann et al. (1986) with a slight modification. Fraction A was concentrated to 1/10 volume with Amicon YM 10 filter. Samples were dialyzed against 10 volumes of 0.5 M urea/0.15 M NaCl/0.05 M Tris-HCl, pH 7.4/2 mM PMSF/0.2% (v/v) CHAPS (buffer B) at 4°C for overnight. CHAPS imprecipitated columns (1% (v/v)) were added to the dialyzed samples, and the dialysate was incubated with gelatin Sephrase 4B gel (1 mL/gel for 1 mg protein) for overnight at 4°C with rotating at 100 rpm. The gel-containing solution was poured into a glass column (1 x 10 cm), and wash with 5 bed volumes of ice cold buffer C. The pass through fractions was collected and concentrated to 1/10 volume with Amicon YM 10 filter and dialyzed against 10 volumes of buffer B without 0.15M NaCl at 4°C overnight.

DEAE-SPW ion-exchange chromatography
DEAE-SPW column was equilibrated with 2M urea/0.05 M Tris-HCl, pH 8.0 at a flow rate of 0.5 mL/min. Samples were subjected to the column, and elution was achieved with an ascending gradient of 2M urea/0.05 M Tris-HCl, pH 8.0: 0-40% in 40 min and 40-100% in 10 min. The elution was monitored by the absorption at 280nm. The distribution of immuno- reactive FN for TN was determined by ELISA.

Enzyme-linked immunosorbent assay (ELISA)
ELISAs were performed according to the method described by Rennard et al. (1980) with a slight modification involving use of labeled avidin-biotin method to increase sensitivity (Yolken et al., 1983). Briefly, the wells of ELISA plate were coated with the serial dilution of the samples to be tested. Biotinylated secondary antibody and peroxidase-conjugated avidin were used at a 1:250 dilution. Incubation time of each step was 1h. PBS/0.5% bovine serum albumin/0.05% Tween 20 was used as washing and dilution buffer. The enzyme substrate (150 mL) [2 mg/ml o- phenylene diamine dihydrochloride in methanol diluted 1:100 into 0.003% (v/v) H2O2] was added. After the color was allowed to generate for 10 min, the reaction was stopped by 2 M H2SO4. The intensity of color produced was measured spectrophotometrically at 492 nm. The values were obtained in the linear range of absorbance. ELISA inhibition test was performed according to the method described by Rennard et al. (1980).

SDS-PAGE and Immunoblotting
Reduced samples were prepared as follows: samples (10-100 mg as protein) were dissolved in 100 mL of 50 mM Tris-HCl pH 6.8/1% (v/v) 2-mercaptoethanol/0.2% (w/v) SDS/20% (w/v) glycerol/0.04% (w/v) bromophenol blue and were heated at 70°C for 30 min. In the case of the preparation of nonreduced samples, 2-mercaptoethanol was omitted from the preparation buffer. Electrophoresis unit was a Dai-ichi Chemical model 1001 vertical slab gel electrophoresis unit. Samples were applied to 4-20% (v/v) SDS polyacrylamide gradient gel. Electrophoresis was at 10mA for 30 min to load samples then 30 mA until the tracking dye was near the end of the gel (Laemmli, U.K., 1970). Gels were fixed in 50% (v/v) methanol/10% (v/v) acetic acid for 2h, stained in 0.025% (w/v) Coomassie Brilliant Blue R 250/25% (v/v) isopropanol/10% (v/v) acetic acid for 2h, and then de-stained in methanol/acetic acid/water (1:1:8 by volume). Immunoblotting was performed by following the procedure of Towbin et al. (1979). TN bands on SDS-PAGE were transferred onto a nitrocellulose sheet with Bio-Rad Trans Blot system at 30 mA for overnight at 4°C. The sheet was washed, incubated with either anti-chicken TN (1:250), or rat anti-human tenasin.
monoclonal antibody (RCB1, 1:100, see below) for overnight at 4°C, and 
successively reacted with peroxidase-conjugated goat anti-rabbit (1:250) or 
peroxidase-conjugated goat anti-rat IgG serum (1:250) for 1h at room 
temperature. 4-Chloro-1-naphtol was used as a substrate for visualization 
of immunoreactive bands.

Hemagglutination

Hemagglutination was carried out by the method described by Yamada 
et al. (1975) using formalinized sheep erythrocytes. Two-fold serial dilutions 
of human fibroblast TN were made starting from 60mg/ml as initial 
concentration.

Electron microscopy of TN

Low-angle shadowing with platinum/carbon was carried out according to 
the method described by Shotton et al. (1979) and Tyler and Branton (1980). 
Samples of ion-exchange column eluent were mixed with an equal volume 
of glycerol, sprayed onto a freshly cleaved mica surface at room temperature 
and dried in the pre-evacuating chamber of an Eiko freeze-fracture and
etching device FD-3 until the chamber reached a vacuum of $2 \times 10^{-7}$ Torr, then in the $2 \times 10^{-1}$ Torr. The dried specimens were shadowed with platinum/carbon on a rotary stage at an angle of 12, then coated with a supporting film of carbon. The replicas were floated on distilled water and picked up on 300-mesh copper grids. Specimens were observed with a Hitachi H7000 electron microscope at 80Kv at a magnification of about x50,000.

**Immunization, cell fusion and cloning**

A 6-weeks-old Wistar female rat was immunized with purified cellular TN (25 μg, emulsified in complete Freund's adjuvant). The injection of TN in incomplete Freund's adjuvant was then repeated four times at weekly intervals. Spleen cells were fused with P3Ag8,653 myeloma line (B-azaguanine-resistant) at a lymphocyte-to-myeloma cell ratio of 5:1, using 42.8% (v/v) polyethylene glycol (Escher et al., 1979). The fusion products were suspended in hypoxantine/aminopterine/thymidine containing medium (RPMI 1640, 20% fcs). The cell suspension was plated (1x10^5 cells/well) into 96-well plates supplemented with feeder cells from peritoneal fluid of a pristin-primed BALB/c mouse and cultured at 37°C. After 2 to 3 weeks of culture the hybridomas showing positive reaction in ELISA on TN were cloned by limiting dilution procedure. Cloning of hybridomas was repeated at least twice. Selected hybridomas clones were stored under liquid nitrogen or grown in larger culture dishes for large scale preparations of monoclonal antibodies. Isootype of monoclonal antibody was determined with the rat monoclonal antibody isotyping kit (Zymet Laboratories).

**ABC Immunohistochemistry (Avidin: biotinylated horseradish peroxidase complex method)**

Tissues obtained from autopsy material were fixed in 10% formalin, embedded in paraffin and cut at 2mm. The sections were deparaffinized in xylene, immersed in 0.3% H₂O₂ in methanol to block endogenous peroxidase activity, washed three times with PBS, incubated in PBS supplemented with 1% normal rabbit serum to block nonspecific binding of immunoglobulins to the tissue sections, incubated in normal goat serum, and then washes in PBS. The monoclonal antibody for 30 min, washed three times with PBS and incubated with a complex of avidin and horseradish peroxidase-conjugated biotin for 30 min. After the sections were washed with PBS, the color reaction was developed with a freshly prepared solution of 0.1% diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in PBS and 0.02% H₂O₂. The sections were then washed in PBS, counterstained with hematoxylin and mounted in Maranon (Muto Chemicals). Culture medium of non-producing P3Ag8,653 mouse myeloma cells was used for control staining.

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**References**


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