Int. J. Dev. Biol. 34: 309-317 (1990)

Original Article

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

YASUTERU OIKE[1], HIDEKI HIRAIWA[2], HISAAKI KAWAKATSU[3], MASAAKI NISHIKAI[4], TSUTOMU OKINAKA[5], TAKANORI SUZUKI[5], AKIKO OKADA[5], RYUICHI YATANI[5] and TERUYO SAKAKURA[6]*

[1]*Japan Immunoresearch Laboratories Inc., Takasaki,* [2]*Amano Pharmaceutical Co., Ltd., Nagoya,* [3]*Nippon Shinyaku Co., Ltd., Kyoto,.* [4]*University of Tokyo, Faculty of Science, Department of Biophysics and Biochemistry, Tokyo,* [5]*Mie University School of Medicine, Department of Pathology, Tsu* and [6]*RIKEN, Laboratory of Cell Biology, Tsukuba, Japan*

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, endosteum, 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-asparatic 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, phenylmethanesulfonyl fluoride; CHAPS, (3-[(3-Cholamidopropyl) dimethylammonio]1-propanesulfonate); SDS-Page, sodiumdodecyl sulfate polyacrylamide gelelectrophoresis; PBS, phosphate buffered salin; BCA, bicinchoninic acid.

*Address for reprints: Laboratory of Cell Biology, RIKEN, Tsukuba, Ibaraki 305, Japan. FAX: 298-36-9010.

0214-6282/90/$02.00

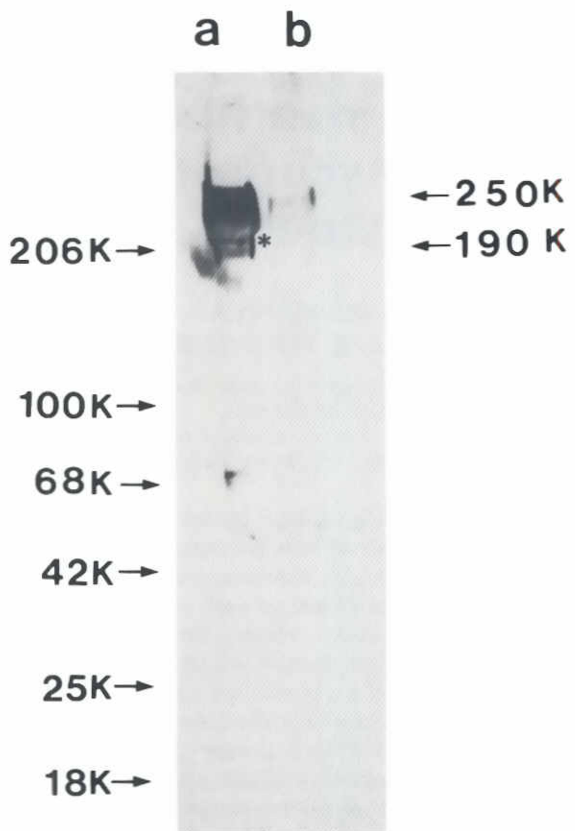

Fig. 1. Immunoblotting of TN sample from HUCF-P2 human fibroblast culture. (a) *2M urea extract (20μg as BCA protein) and* **(b)** *4M guanidinium chloride extract (5μg as BCA protein) run on a 4%-20% SDS-PAGE under reducing condition and transferred onto a nitrocellulose sheet. TN bands were reacted with rabbit anti-chicken TN antibody. Arrows on the left side of this Fig. indicate the positions of molecular weight marker. Arrows on the right indicate major tenascin subunits. The band indicated by * is a minor band (molecular weight 210k) which was occasionally observed in fibroblast TN.*

migration pathways of embryonic neural crest cells (Thesleff *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 immunohistochemical 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[1] 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.

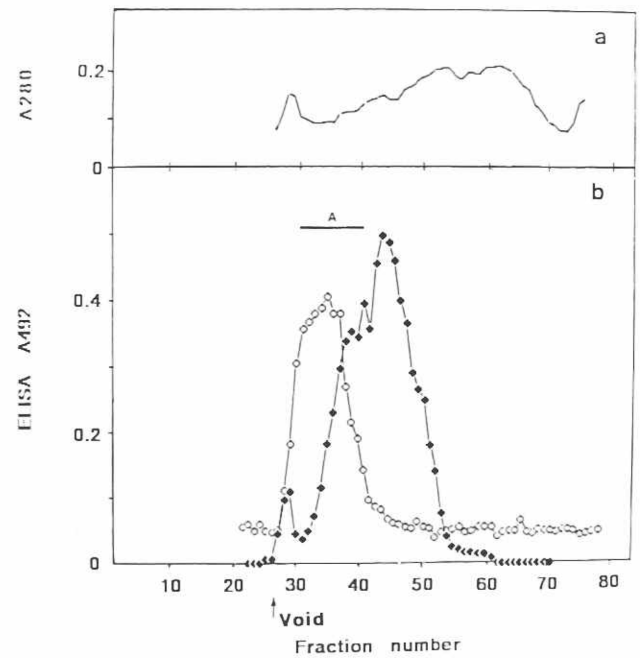

Fig. 3. Distribution of protein and antigenic profile of TN and FN on a Sepharose CL4B column chromatography. *One hundred mg of 2M urea extract applied on a Sepharose CL4B column and eluted.* **(a)** *280 nm absorbance.* **(b)** *Distribution of immunogenicity of TN (○–○) and of FN (●–●).*

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).

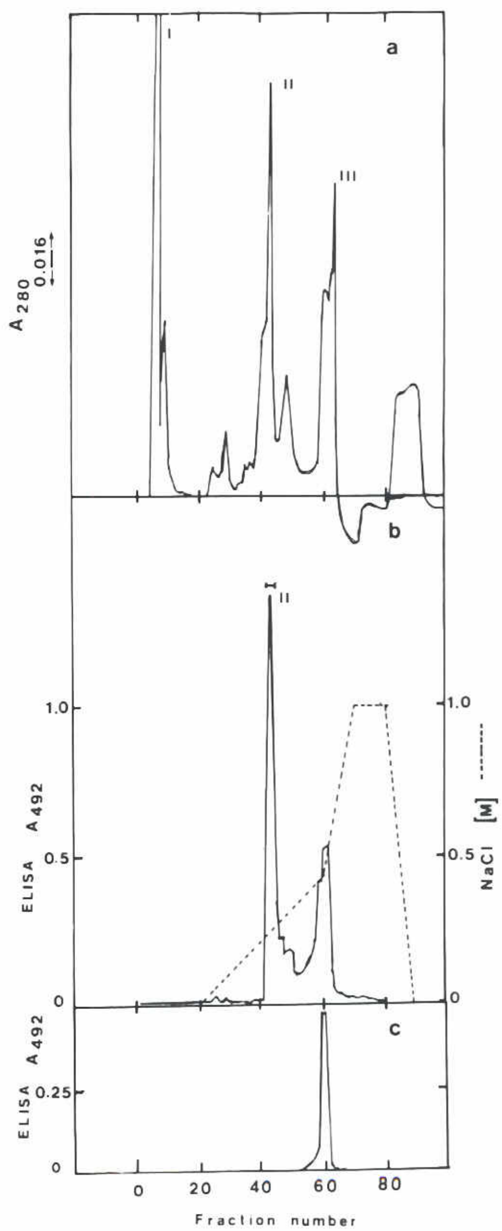

Fig. 4. DEAE-5PW ion-exchange column chromatography of Fraction A after gelatin Sepharose CL4B chromatography. *Elution was done under the conditions described in the text and monitored at 280 nm absorbance* **(a)**. *Distribution of immunogenicity against TN* **(b)** *and chondroitin sulfate* **(c)**.

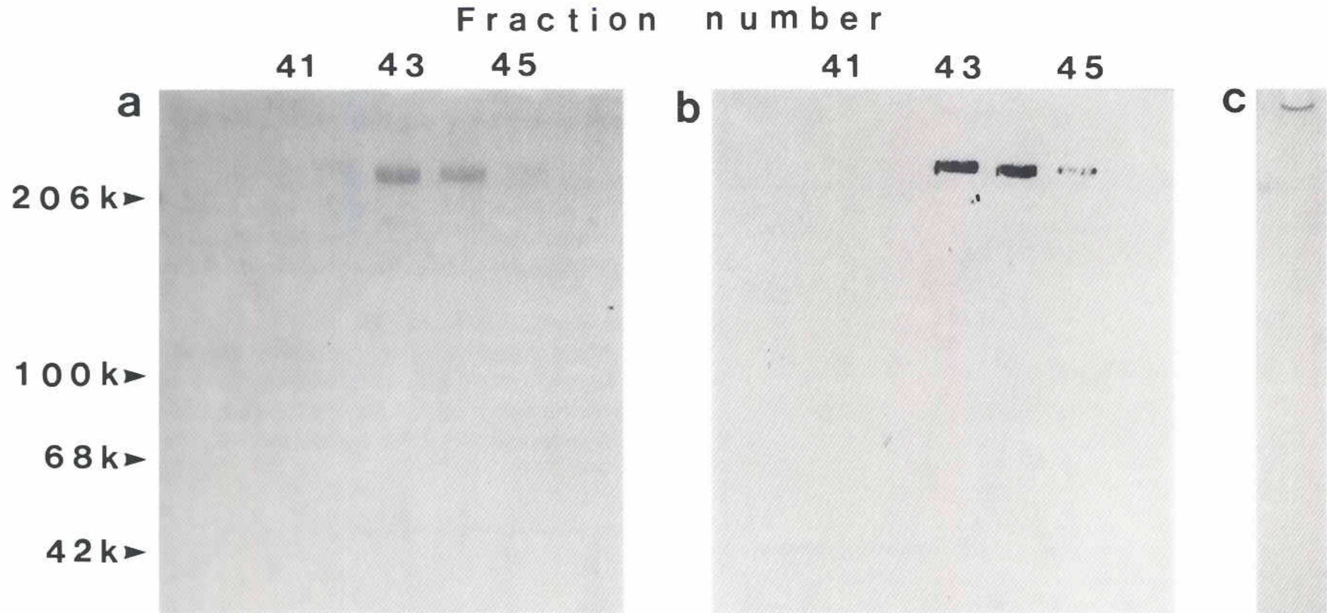

Fig. 5. SDS-PAGE analysis and Immunoblotting of Fraction II. (a) *SDS-PAGE of the DEAE-5PW ion-exchange column fractions (numbers 40 to 46 shown in Fig. 4a) under reducing condition and* **(b)** *its Immunoblotting using chick TN polyclonal antibody.* **(c)** *SDS-PAGE of fraction 43 under non-reduced condition. Arrows indicate the positions of the molecular weight.*

Establishment of monoclonal antibodies to human TN

Fifteen hybridoma cell lines that secreted immunogloblins 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 comfirmed 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), periosteum and endosteum 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, cerebellum, 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 most likely proteolytic degradation products. Third, like chicken TN, human TN has high hemagglutinating activity, and this property may be one of

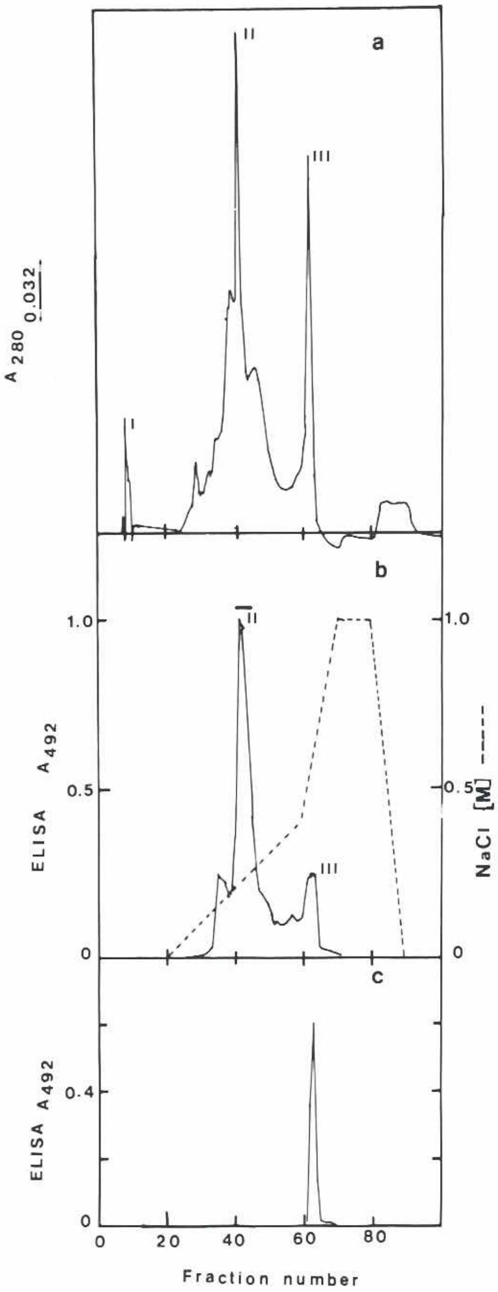

Fig. 6. DEAE- 5PW ion-exchange chromatography of Fraction A from conditioned medium after gelatin Sepharose 4B chromatography. *Elution condition was same as in Fig. 4 and monitored at 280 nm absorbance* **(a)**. *Distribution of immunogenicity of TN* **(b)** *and chondroitin sulfate* **(c)**.

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).

Fig. 7. SDS-PAGE analysis of Fraction II in Fig. 6a. *Five µg of the reduced sample was applied to 4%-20% SDS-PAGE and stained with coomasie briliant blue* (lane a). *Its immunoblotting by anti-chicken TN polyclonal antibody* (lane b). *Arrows indicate the apparent molecular weight of two subunits.*

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. DMEM1, 1:1 mixture of DMEM and Ham F-12 culture medium, PMSF1, CHAPS1, anti-chondroitin sulfate antibody (clone CS56), RPMI 1640 culture medium containing hypoxantin/aminopterine/thymidine, RPMI 1640 containing hypoxantine/thymidine, polyethylene glycol (molecular weight 3,800) and pristan were from Sigma; fetal calf serum (fcs)1 was from Grand Island Biological Co.; cell culture bottles and ELISA plates (3912) were from Beckton and Dickinson; biochemical grade urea and ammonium sulfate were from Wako Pure Chemical Co.; 1BCA protein assay reagents were from Piearce. 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-

Fig. 8. Hemagglutination of formalinized sheep erythrocytes by human TN purified from cultured fibroblasts. *Two-fold serial dilutions of TN were made starting from 60 µg/ml as the initial concentration. The minimal agglutinating concentration was 7.5 µg/ml (arrow).*

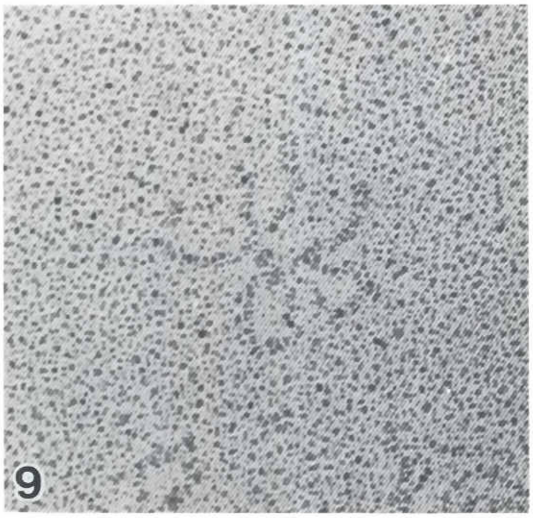

Fig. 9. Electron microscopic images of purified human TN. *TN exhibits a six-armed structure with a central globule. (x270,000)*

acrylamide gel plates (4%-20%) were from Dai-ichi Pure Chemicals; prestained 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 Toso. Other chemicals for SDS-PAGE[1] were from Bio-Rad Japan. Nitrocellulose membrane (Nitro

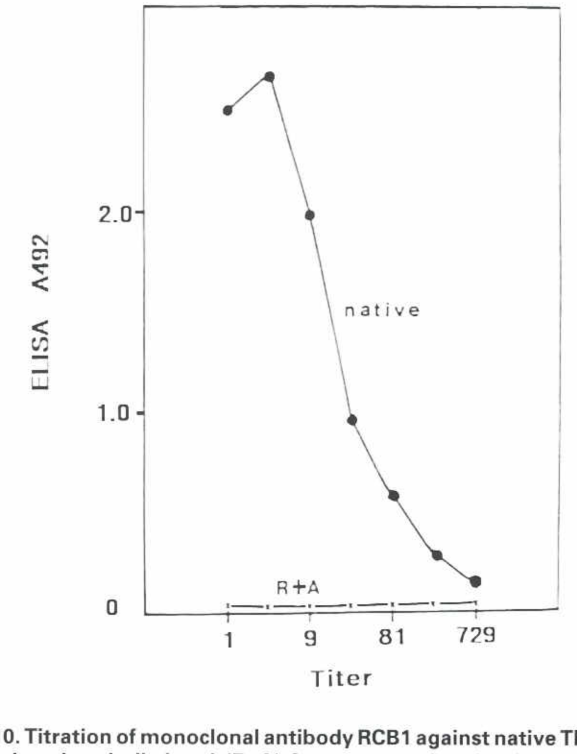

Fig. 10. Titration of monoclonal antibody RCB1 against native TN and its reduced and alkylated (R+A) form *prepared under the condition described elsewhere (Kimata et al., 1978). Each of samples (0.2 µg of BCA protein per well) were coated by native (•–•) and (R+A) (x–x) TN. ELISA was done under the conditions described in the text. Note that immunogenicity of TN against RCB1 was completely destroyed by the reduction and alkylation.*

Fig. 11. Immunoblotting by RCB1. *Aliquots of 2M urea extract were run on SDS-PAGE. Protein bands were transferred onto nitrocellulose sheet. TN bands were visualized by ELISA using RCB1.*

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 cm^2 roller bottles at a density of $2x10^5$ 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, (l984). 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 (In preliminary experiment 33.3% ammonium sulfate was found to precipitate TN efficiently

TABLE 1

MONOCLONAL ANTIBODY RCB1 REACTIONS WITH HUMAN TISSUES

Tissue	No. of cases	No. of positive(%)	Positive area
Normal			
Brain			
Cerebrum	24	6 (25.0%)	glia
Cerebellum	11	0	
Digestive organs			
Esophagus	29	21 (72.4%)	lamina propria, muscle layer
Stomach	38	28 (73.6%)	muscularis mucosae, muscle layer, perineurium of plexus mesentericus
Intestine	39	31 (79.5%)	lamina propria, muscularis mucosae, muscle layer, perineurium of plexus mesentericus
Colon	38	26 (68.4%)	muscle layer, muscularis mucosae perineurium of plexus mesentericus
Liver	42	9 (21.4%)	sinusoid
Pancreas	42	0	
Gall bladder	35	13 (37.1%)	muscle layer, lamina propria
Salivary	11	0	
Hematopoetic organs			
Bone marrow	26	26 (100 %)	periosteum, endostenm
Spleen	38	10 (26.3%)	trabecula, cord
Lymph node	37	30 (71.4%)	capsule, trabecula
Endocrine organs			
Pituitary	4	4 (100%)	intercellular space
Thyroid	45	0	
Adrenal	38	26 (81.1%)	interstitium of medula
Oary	21	1 (4.8%)	corpus luteum
Testis	25	0	
Urogenital organs			
Kidney	49	49 (100 %)	interstitium of distal tubule
Urinary bladder	18	11 (61.1%)	smooth muscle
Cervix	20	14 (70.0%)	lamina propria
Prostate	15	12 (80.0%)	periductal connective tissue
Others			
Lung	48	30 (62.5%)	lamina propria of bronchus, perichondrium alveolar wall
Skin	24	9 (37.5%)	subepithelial layer
Placenta	2	0	
Breast	5	0	
Heart	42	0	
Neoplastic			
Pulmonary cancer	8	8	
gastric cancer	2	1	
Esophageal cancer	2	2	
Colon cancer	3	2	connective tissues surrounding the cancer nests
Pancereatic cancer	3	3	
Hepatoma	4	2	
Phayrngeal cancer	1	1	
Lymphoma	2	2	
Glioma	1	1	
Uterine cancer	1	1	

(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

Sepharose CL4B chromatography: Crude TN fraction from either cells or conditioned media was applied to Sepharose CL4B column (2.5 x 110 cm) which had been equilibrated with 2M urea/0.15 M NaCl/0.05M Tris HCl pH 7.4/2 mM PMSF/0.2% (w/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 20 ml/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% w/v CHAPS (buffer C) at 4°C for overnight. CHAPS improved column recovery of TN. The dialyzate was incubated with gelatin Sepharose 4B gel (1ml/gel for 1mg protein) for overnight at 4°C with rotating at 100 rpm. The gel-containing solution was poured into a glass column (1x10cm), and washed 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-5PW ion-exchange chromatography

DEAE-5PW column was equilibrated with 2M urea/0.05 M Tris HCl, pH8.0 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/1 M NaCl/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 immunogenicity for TN was determined by ELISA.

Enzyme-linked immunoadsorbent assay (ELISA)[1]

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. Anti-chicken TN at 1:250 and anti-chondroitin sulfate at 1:250 were used as the first antibodies. Biotinylated second antibody and peroxidase-conjugated avidin were used at a 1:250 dilution. Incubation time of each step was 1h. PBS[1]/0.5% bovine serum alubmin/0.05% Tween 20 was used as washing and dilution buffer. The enzyme substrate (150 ml) [2 mg/ml o-phenylene diamine hydrochloride in methanol diluted 1:100 into 0.003% (v/v) H_2O_2] was added. After the color was allowed to generate for 10 min, the reaction was stopped by 2 M H_2SO_4. 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 (10-100mg as protein) were dissolved in 100 µl of 50 mM Tris HCl pH 6.8/1% (v/v) 2-mercaptoethanol/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 100 I vertical slab gel electrophoresis unit. Samples were applied to 4-20% (w/v) SDS polyacrylamide gradient gel. Electrophoresis was at 10 mA 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) Coomasie Billiant Blue R 250/25% (v/v) isopropanol/10% (v/v) acetic acid for 2h, and then destained 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 tenascin

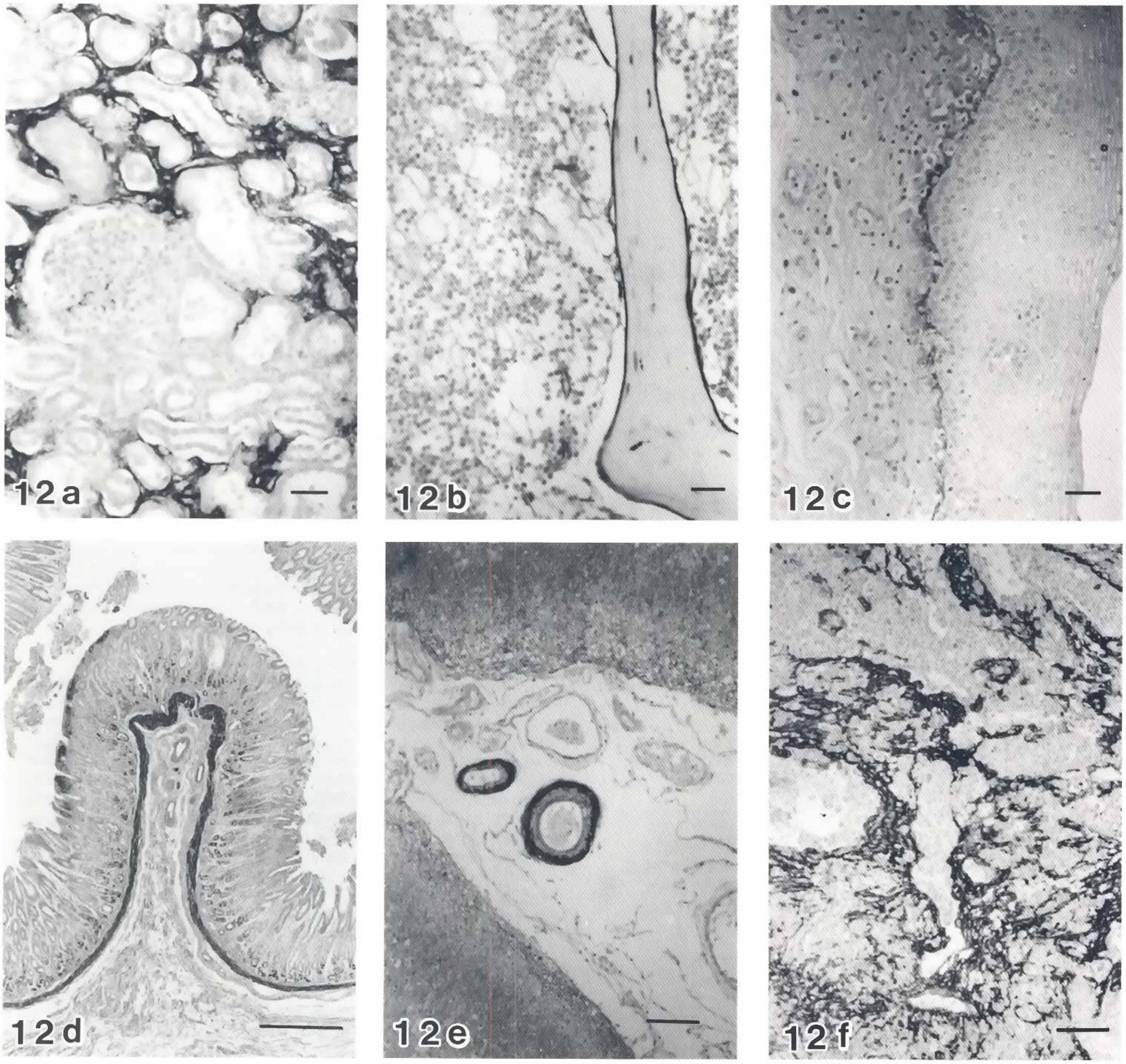

Fig. 12. Immunoperoxidase reaction on various human tissues. *RCB1 reacts with interstitium of renal distal tuble* **(a)**, *periosteum and endosteum of bone marrow* **(b)**, *lamina propria of esophagus* **(c)**, *muscularis mucosae of stomach* **(d)**, *media of arteries of subarachnoid* **(e)** *and dense mesenchyme surrounding cancer nests* **(f)**. *Scale bars: a, b, c, e, and f=100μm; d=50μm*

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 suface 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 2×10^{-2} Torr, then in the 2×10^{-7} 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 Wister 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 P3xAg8,653 myeloma line (8-azaguanine-resistant) at a lymphocyte-to-myeloma cell ratio of 5:1, using 42.8% (v/v) polyethylene glycol (Eshhar *et al.*, 1979). The fusion products were suspended in hypoxantine/aminopterine/thymidine containing medium (RPMI 1640, 20% fcs). The cell suspension was plated (1×10^5 cells/well) into 96-well plates supplemented with feeder cells from peritoneal fluid of a pristan-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 hybridoma was repeated at least twice. Selected hybridoma clones were stored under liquid nitrogen or grown in larger culture dishes for large scale preparations of monoclonal antibodies. Isotype 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 deparaffined in xylene, immersed in 0.3% H_2O_2 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 with the hybridoma culture fluid containing the monoclonal antibody for 60 min, washed three times with PBS, incubated with rat immunoglobulins 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% H_2O_2. The sections were then washed in PBS, counterstained with hematoxylin and mounted in Marinol (Muto Chemicals). Culture medium of nonproducing P3xAg8,653 mouse myeloma cells was used for control staining.

Acknowledgments

The authors would like to thank Dr. K. Kimata for critical reading of the manuscript and Drs. R.A. Shiurba, Y. Yoshinaka, Y. Tomooka and M. Kusakabe for valuable advice on biochemical procedures, cell cultures and immunohistochemistry, Mr. J. Morita for the preparation of tissue sections and Mrs. I. Onuma for the preparation of manuscript. This work was partially supported by Grants from the Ministry of Education, Science and Culture of Japan.

References

AUFDERHEIDE, E., CHIQUET-EHRISMAN, R. and EKBLOM, P. (1987). Epithelio-mesenchymal interaction in the developing kidney leads to expression of tenascin in the mesenchyme. *J. Cell Biol. 105:* 599-609.

BOURDON, M.A., WIKSTRAND, C.J., FURTHMAYR, H., MATTHEWS, T. J. and BIGNER, D.D. (1983). Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. *Cancer Res. 43:* 2796-2805.

CHIQUET, M. and FAMBROUGH, D.W. (1984). Chick myotendinous antigen. 1.A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol. 98:* 1926-1936.

CHIQUET-EHRISMANN, R., KALLA, P., PEARSON, C.A., BECK, K. and CHIQUET, M. (1988). Tenascin interferes with fibronectin action.*Cell 53:* 383-350.

CHIQUET-EHRISMANN, R., MACKIE, E.J., PEARSON, C.A. and SAKAKURA, T. (1986). Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell 47:* 131-139.

ERICKSON, H.P. and INGLESIAS, J.L. (1984). A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature 311:* 267-269.

ERICKSON, H.P. and TAYLOR, H.C. (1987). Hexabrachion proteins in embryonic chicken tissues and human tumors. *J. Cell Biol. 105:* 1387-1394.

ESHHAR, Z., BLUTT, C., BERGMAN, Y. and HAIMOVICH, J. (1979). Induction of secretion of IgG from cells of the B cell line 38C-13 by somatic cell hybridization. *J. Immunol. 122:* 2430-2433.

FRIEDLANDER, D.R., HOFFMAN, S. and EDELMAN,G.M. (1988). Functional mapping of cytotactin: proteolytic fragments active in cell-substrate adhesion. *J. Cell Biol. 107:* 2329-2339.

INAGUMA, Y., KUSAKABE, M., MACKIE, E.J., PEARSON, C.A., CHIQUET-EHRISMAN, R. and SAKAKURA, T. (1988). Epithelial induction of stromal tenascin in the mouse mammary gland: from embryogenesis to carcinogenesis. *Dev. Biol. 128:* 245-255.

KIMATA, K., OIKE, Y., ITO, K., KARASAWA, K. and SUZUKI, S. (1978). The occurrence of low buqyant density proteoglycans in embryonic chick cartilage. *Biochem. Biophys. Res. Com. 85:* 1431-1439.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature 227:* 680-685.

MACKIE, E.J., CHIQUET-EHRISMAN, R., PEARSON, C.A., INAGUMA,Y., TAYA, K., KAWARADE, Y. and SAKAKURA, T. (1987). Tenascin is a stromal marker for epithelial malignancy in the mammary gland. *Proc. Natl. Acad. Sci. U.S.A. 84:* 4621-4625.

RENNARD, S.I., KIMATA, K., WILCZEC, J., KIMURA, J.H. and HASCULL,V.C. (1980). An enzyme-linked immunoassay for the cartilage proteoglycan. *Arch. Biochem. Biophys. 207:* 399-406.

SHOTTON, D.M., BURBE, B.E. and BRANTON, D. (1979). The molecular structure human erythrocyte spectrin. *J. Mol. Biol. 131:* 303-329.

SMITH, P.K., KROHN, R.I., HERMANSON, C.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. and KLENK, D.C. (1985). Measurement of protein using bicinchoninic acid. *Ann. Biochem. 150:* 76-85.

TAKEDA, H., OIKE, Y. and SAKAKURA, T. (1988). Immunofluorescent localization of tenascin during development of the mouse urogenital sinus: possible involvement in genital duct morphogenesis. *Differentiation 39:* 131-138.

THESLEFF, I., MACKIE, E., VAINIO, S. and CHIQUET-EHRISMAN, R. (1987). Changes in the distribution of tenascin during tooth development. *Development 101:* 289-296.

TOWBIN, H., STAEHELIN, J. and GORDON, H. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:* 4350-4354.

TYLER, J.M. and BRANTON, D. (1980). Rotary shadowing of extended molecules dried from glycerol. *J. Ultrastruct. Res. 71:* 95-102.

YAMADA, K.M. and AKIYAMA, S.K. (1984). Preparation of cellular fibronectin. In *Cell Culture Methos for Molecular and Cell Biology, Vol. 1. Methods for Preparation of Media, Supplement and Substrate for Serum-Free Animal Cell Culture* (Eds. B.W. Barns, D. Sirbask and G.W. Sato). Alan R. Liss, New York, pp.215-230.

YAMADA, K.M., YAMADA, S.S. and PASTAN, I (1975). The major cell glycoprotein of chick embryo fibroblasts in an agglutinin. *Proc. Natl. Acad. Sci. USA 72:* 3158-3612.

YOLKEN, R., LEISTER, F., WHITCOMB, L. and SANTOSHAM, M. (1983). Enzyme immunoassays for the detection of bacterial antigens utilizing biotin-labeled antibody and peroxidase biotin-avidin complex. *J. Immunol. Method. 56:* 319-327.

Accepted for publication: March 1990