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Human Carcinoma Cells Synthesize and Secrete Tenascin *in vitro*

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Tenascin is an extracellular matrix glycoprotein produced in response to epithelial-mesenchymal interactions that initiate fetal organogenesis, and it is also found in the stroma of benign and malignant neoplasms. Thirty-five human cell lines representing a variety of cancers were examined by immunoprecipitation and polyacrylamide gel electrophoresis of radiolabeled tenascin proteins from conditioned media. Two forms of tenascin with relative molecular masses of 190,000 and 250,000 were identified. Eight cell lines produced both forms. With the exception of myeloid and lymphoid leukemias and Burkitt's lymphoma, all of the mesodermal and neuroectodermal tumor lines were found to synthesize tenascin. Unexpectedly, tenascin was secreted by several mammary and colonic adenocarcinomas as well as by a line derived from normal mammary epithelial cells, and in some cases increased production was induced by transforming growth factor beta in serum-free medium. Cells producing fibronectin but not tenascin attached and spread on plastic culture dishes, while those producing tenascin alone remained suspended in the medium or were rarely attached. Tenascin also inhibited fibronectin-mediated adhesion of MCF7 breast carcinoma cells *in vitro*. The results suggest that tenascins synthesized and secreted by some cancer cells, especially those of epithelial origin, may have specific roles in determining tumor cell adhesion and ultimately the ability to form invasive outgrowths.

Key words: Tenascin — Human carcinoma cell

Tenascin (TN[6]) is an extracellular matrix (ECM) glycoprotein with a unique six-armed macromolecular structure[1,2] that is known to be an essential factor for modulation of reciprocal epithelial-mesenchymal interactions during fetal organogenesis.[3–8] The relative abundance of TN gradually declines in the ECM after birth, but it increases again in the stroma of malignant tumors where it has been hypothesized to play critical roles affecting cancer cell adhesion, migration, and proliferation.[1,9,10] Production of TN by mesenchymal stromal cells is induced as a result of cell-cell interactions with embryonic or malignant epithelium.[5,8–10] Immunohistochemical staining has revealed TN in the ECM of dense connective tissues in developing epithelial organs such as mammary gland[1,10] and in that surrounding smooth muscle cells of the digestive tract.[8,11] It is also deposited within nests of cancer cells,[1,9–11] but whether it is synthesized by tumor cells as well as normal cells in these sites is unknown. Likewise, the pathological functions of TN in tumorigenesis, local invasion, and metastasis remain elusive. *In vitro,* chick TN stimulates the growth of rat mammary tumor cells,[1] a bioactivity that may be related to the fact that the primary structure of TN contains multiple repeats of amino acid sequences resembling epidermal growth factor (EGF).

Several forms of TN with differing molecular weights are known to appear sequentially during the development of some tissues such as gut, and their expression depends on epithelial-mesenchymal interactions.[8] The gene encoding chick "cytotactin/tenascin" was the first to be cloned and sequenced,[12,13] and recently the complete sequences of the mouse and human TN genes as well as their deduced amino acid sequences have been reported.[14–16] The data show a TN subunit protein composed of several domains that contain EGF-like repeats, fibronectin (FN) type III-like repeats, and fibrinogen-like sequences with minor differences between species. In addition, analysis of recombinant TN fragments indicates that there are separate domains with contrary functions, one for cell adhesion and another for repulsion.[2] These may influence the rate and direction of cell movement in tissues. Moreover, TN precursor mRNA undergoes alternative splicing to produce isoforms that have numerous poten-

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[6] The abbreviations used are: TN, tenascin; ECM, extracellular matrix; FN, fibronectin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGF-β, transforming growth factor beta; EGF, epidermal growth factor; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; GMEM, glioma-mesenchymal extracellular matrix antigen; M_r, relative molecular mass; kDa, kilodalton.

tial N-linked glycosylation sites. Consequently, polymorphism of TN isoforms may derive from both post-transcriptional splicing of precursor mRNA and from post-translational modification of the polypeptide by carbohydrates.

These properties suggest that TN is a multifunctional macromolecule that may be a key ECM component for growth regulation of epithelial and mesenchymal tissues. Identification of the various isoforms of TN as well as the glycosylation patterns that distinguish them represents a major challenge for ultimately understanding their biological functions in normal development and cancer. The present survey was undertaken in order to begin an analysis of TN synthesis by human cancer cells. TN glycoproteins from a variety of malignant cell lines were isolated by immunoprecipitation of conditioned medium and cell lysates, and they were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Here we report the results including the unexpected finding that TN was synthesized and secreted by normal and malignant epithelial cells *in vitro*, probably affecting the biological behavior of the cells.

MATERIALS AND METHODS

Cell lines GOTO was from Dr. Imanishi, Kyoto Prefecture Medical University (Kyoto). LX-I was from Dr. Tashiro, Cancer Institute (Tokyo), and U251 MG was from Dr. Takahashi, Aichi Cancer Center Research Institute (Nagoya). The remainder of the cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Antibodies and analytical reagents A polyclonal antibody against human TN was raised in a female rat according to standard procedures. Briefly, TN (10–100 μg), purified from human fibroblast cells (HUCF-P2)[11] was emulsified with Freund's complete adjuvant, and it was injected s.c. For boosting, TN was emulsified with incomplete adjuvant and was injected three times as two-week intervals. Blood was collected from the tail vein, and immune sera preabsorbed with human FN were stored at -80°C. Human TN purified from A375 melanoma cells was purchased from JIMRO (Takasaki). Mouse FN was obtained from UCB-Bioproducts (S.A., Belgium). Bovine serum albumin (BSA, fraction V) was from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated goat anti-rat IgG and rabbit anti-human FN antiserum (Cat. No. 0201-0832) were from Cappel (West Chester, PA). Protein A-Sepharose CL-4B (Cat. No. 17-0963-03) was from Pharmacia (Uppsala, Sweden).

Radiolabeling of proteins in cell culture Cell lines were maintained in Eagle's modified minimal essential medium (MEM) supplemented with Earle's salts and 10% fetal calf serum (FCS). In some cases, serum-free Dulbecco's modified Eagle's/F12 medium supplemented with transforming growth factor beta (TGF-β, 100 ng/ml), mouse EGF (10 ng/ml), bovine insulin (10 μg/ml), human transferrin (10 μg/ml), and sodium selenite (10^{-8} *M*) was used. TGF-β and EGF were from Toyobo (Tokyo). All other cell culture reagents including media, penicillin, streptomycin, bovine insulin, human transferrin, and sodium selenite were from Sigma Chemical Co. When cells were in mid-log phase, media were changed to methionine-free medium, and cells were labeled with [^{35}S]methionine (50 mCi/ml, SJ1015 > 1000 Ci/mmol, Amersham, UK) for 12 to 18 h. Media were collected, and in some cases cells were lysed with buffer A (150 m*M* NaCl, 0.01 *M* disodium ethylenediaminetetraacetate, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton X-100, 20 m*M* NaF, 0.00025 *M* phenylmethylsulfonyl fluoride, 5 μmg/ml leupeptin, and 50 m*M* Tris-HCl, pH 8.2). Conditioned media and cell lysates were then immunoprecipitated with anti-TN and anti-FN antibodies.

Immunoprecipitation Polyclonal antibodies (2 μl) that specifically recognized TN or FN were added to separate aliquots of [^{35}S]methionine-labeled medium (3×10^6 cpm) from each cell line, and samples were incubated for 3 h at 4°C with gentle agitation. Lysates of four cell lines (MCF7, HBL100, W138, and HT1080) also were treated with anti-TN antibody. Then 200 μl of protein A-Sepharose CL-4B solution (10% v/v with buffer A) was added to each sample, and they were incubated for 1 h at 4°C. After centrifugation, the antigen-antibody-protein A-Sepharose complexes were washed by resuspending and centrifuging four times in buffer A followed by one time in phosphate-buffered saline (PBS), pH 7.2. Proteins were eluted from the resins by incubation with 40 μl of sample buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 0.1 *M* dithiothreitol, and 50 m*M* Tris-HCl, pH 6.8) for 2 min at 100°C, and they were electrophoretically separated on gradient gels (4–20%) at 30 mA constant current. Radioactive bands were visualized by fluorography. For higher resolution, 5% SDS-PAGE was used. Rainbow ^{14}C-labeled protein markers (Cat. No. CFA-756) from Amersham (Tokyo), including myosin (M_r 200,000), phosphorylase B (M_r 92,500), BSA (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,300), were used as molecular weight markers.

Western blot analysis After SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene-difluoride membranes (PVDF, IPVH 000-10) from Millipore (Bedford, MA) according to the method of Towbin *et al.*[17] with a minor modification. Briefly, transfer was completed within 15 h at 30 mA constant current. After blocking of non-specific protein binding sites with PBS containing 1% BSA and 0.05% Tween-20 for 30 min,

membranes were incubated in primary antibody solution (1:400) for 3 h at room temperature. Then membranes were washed three times in blocking solution, and immersed in a peroxidase-conjugated secondary goat anti-rat IgG (1:400) for 1 h. After three more washes, immunoreactive proteins were visualized by immersion of the membranes in a chromogen solution containing 4-chloro-1-naphthol (10 mg dissolved in 10 ml of methanol, to which 40 ml of deionized water was added) for 15 min.

Cell-substrate adhesion All thirty-five cell lines were used for this experiment. In serum-free Eagle's MEM, cells from each line were seeded at 10^5/ml into plastic dishes (Falcon 3002), and they were cultured for 3 h or 18 h under standard conditions at 37°C in a CO_2 incubator. After 3 h, media were removed, and the dishes were gently rinsed three times with PBS. Cell attachment was evaluated by phase-contrast microscopy. Cells were either polygonal or round in shape, and they were classified as suspended in the medium, attached, or spreading on the substrate. The ability of TN to inhibit FN-mediated spreading of MCF7 was also tested. Plastic culture dishes were precoated for 1 h at room temperature with a 13 n*M* solution of FN alone, TN alone, BSA alone, FN/TN (1:1), or FN/BSA (1:1). Dishes were treated with heat-denatured BSA to reduce non-specific binding of cells as described previously.[18] MCF7 cells were then plated on the dishes, and they were incubated at 37°C. After 1 h, the cells were stained with toluidine blue and photographed.

RESULTS

Production of tenascin and fibronectin by human cell lines Antibody specificities were determined by Western blot analysis using purified TN[11] and FN (by-product of TN purification) as antigens. No cross-reactivities were found between the antibodies (Fig. 1). A wide variety of human cancer cell lines were examined by screening of conditioned media for TN and FN by immunoprecipitation (Table I). Among thirty-five lines tested, fourteen were immunoreactive with TN antibody. Positive cells included not only those of mesodermal and neuroectodermal origin but also some that were derived from epithelium. Of the latter, there were two mammary adenocarcinomas (MDA-MB-231, MDA-MB-468), a normal mammary line (HBL100), and 4 colonic adenocarcinomas (SW480, SW620, COLO205, COLO320DM). As examples, the expression patterns of ECM proteins in three mammary cell lines are shown in Fig. 2. In some cases, an increase in the level of TN synthesis was also inducible with TGF-β. When the normal mammary line HBL 100 and the colon carcinoma line COLO205 were grown in serum-free medium, immunoprecipitable ^{35}S-labeled TN was detected, and production, particularly

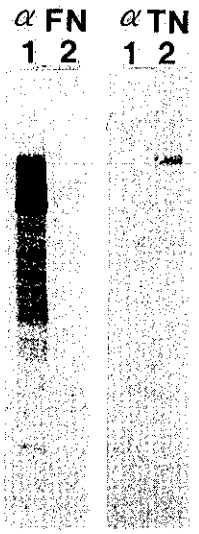

Fig. 1. Antibody specificities. Western blots of FN (1) and purified TN (2) stained with anti-FN and anti-TN polyclonal antibodies showed no cross-reactivities.

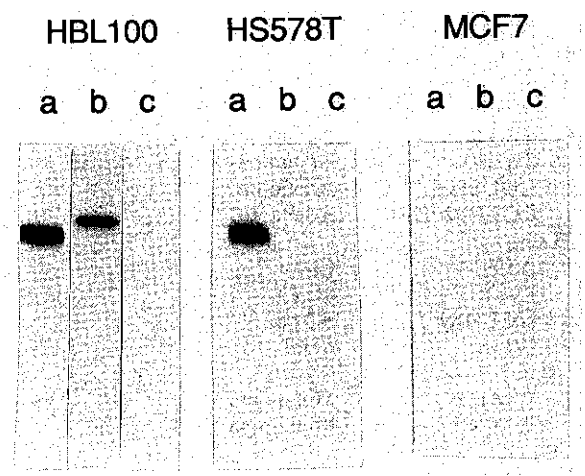

Fig. 2. Immunoprecipitable FN (lane a) and TN (lane b) in FCS-containing conditioned media from normal (HBL100) and malignant (HS578T, MCF7) mammary epithelial cell lines. Normal rabbit serum control (lane c).

of M_r 220,000–230,000 TN in HBL 100, was increased in responseto TGF-β. By contrast, colon carcinoma line COLO320 synthesized TN at relatively high levels in serum-free medium, but no increase was observed in the presence of TGF-β (Fig. 3, lanes c). Lysates from the cells lines tested with anti-TN antibody (MCF7, HBL-100, WI38, and HT1080) showed only traces of immunoprecipitable TN.

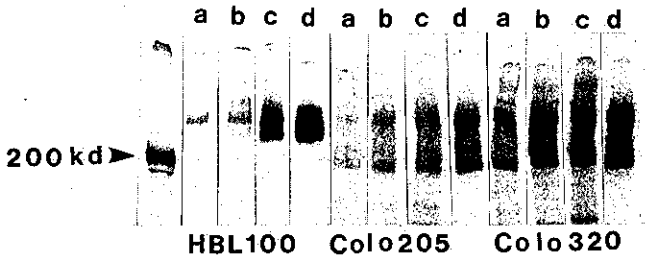

Fig. 3. Immunoprecipitable TN in serum-free conditioned medium from normal mammary cell line HBL100 and colonic adenocarcinoma cell lines COLO205 and COLO320. Cells were grown in serum-free Dulbecco's modified Eagle's/F12 medium plus the following supplements: selenium (lane a); insulin, transferrin, EGF, and selenium (lane b); insulin, transferrin, EGF, selenium, and TGF-β (100 ng/ml) (lane c); and medium containing 10% FCS (lane d).

Table I. Immunoprecipitable Tenascin and Fibronectin in Conditioned Medium from Human Cancer Cell Lines[a]

Cell line	Type	TN		FN	Adhesion to plastic substrate[b]	
		250 kDa	190 kDa		3 h	18 h
Breast						
MCF7	Adeno ca	−	−	−	s	s
T47D	Ductal ca	−	−	−	s	s
DU4475	Met ca	−	−	−	s	s
ZR-75-1	Ductal ca	−	−	−	s	s
HBL100	Normal	+	+/−	+	sp	sp
MDA-MB-231	Adeno ca	+	−	+	r/sp	r/sp
MDA-MB-468	Adeno ca	+	−	+	r	r
HS578T	Ductal ca	−	−	+	sp	sp
SK-BR-3	Adeno ca	−	−	+	r	r
Colon						
WiDr	Adeno ca	−	−	−	s	r/sp
SW403	Adeno ca	−	−	−	s/r	s/r
SW480	Adeno ca	+	+	−	s	r
SW620	Adeno ca	+	+	−	s	r/sp
COLO205	Adeno ca	+	+	−	s	s
COLO320DM	Adeno ca	+	+	−	s	r
Lung						
A549	Ca	−	−	+	sp	sp
LX-1	SC ca	−	−	−	s	s
NCI-H69	SC ca	−	−	−	s	r
WI38	Fetal	+	−	+	r	r/sp
Skin						
WM115	Melanoma	+	+	+	sp	sp
WM266-4	Melanoma	+	+	+	sp	sp
Blood						
J111	Leukemia	−	−	−	sp	sp
K562	Leukemia	−	−	−	s	s
HL60	Leukemia	−	−	−	s	s
MOLT4	Leukemia	−	−	−	s	s
CCRF-CEM	Leukemia	−	−	−	s	s
Other						
Namalwa	Burkitt's lymphoma	−	−	−	s	s
Hep2	Epidermoid ca	−	−	−	r/sp	r/sp
A431	Epidermoid ca	−	−	−	s	s/r
Chang	Normal liver	−	−	+	s	r
HepG2	Hepatoma	−	−	+	sp	sp
PA-1	Teratocarcinoma	+	−	+	r/sp	r
HT1080	Fibrosarcoma	+	+/−	+	r/sp	r/sp
GOTO	Neuroblastoma	+	−	+	s	s/r
U251	Glioblastoma	+	−	+	sp	sp

a) Immunoprecipitable TN and FN in FCS-containing conditioned medium: + =strong; +/− = weak; − =negative.
b) Cell-substrate adhesion: s, suspended polygonal; r, round; sp, spreading polygonal.

Table II. Adhesion of Cancer Cell Lines to Plastic Substrate after 3 h and 18 h in Serum-free Medium

ECM component	No. of cell line	Polygonal suspended		Round						Polygonal spreading	
				Suspended		Attached		Spreading			
		3 h	18 h	3 h	18 h	3 h	18 h	3 h	18 h	3 h	18 h
None	16	13	10	1	2	0	1	1	2	1	1
TN	4	4	1	0	0	0	2	0	1	0	0
FN	5	1[a]	0	0	0	1	2	0	0	3	3
TN+FN	10	1	0	0	1	2	2	3	3	4[b]	4[b]

All thirty-five cell lines were tested.
a) SK-BR-3 breast adenocarcinoma cells expressed only FN.
b) WM115 and WM266-4 melanoma cells expressed both 250 kDa and 190 kDa TN as well as FN.

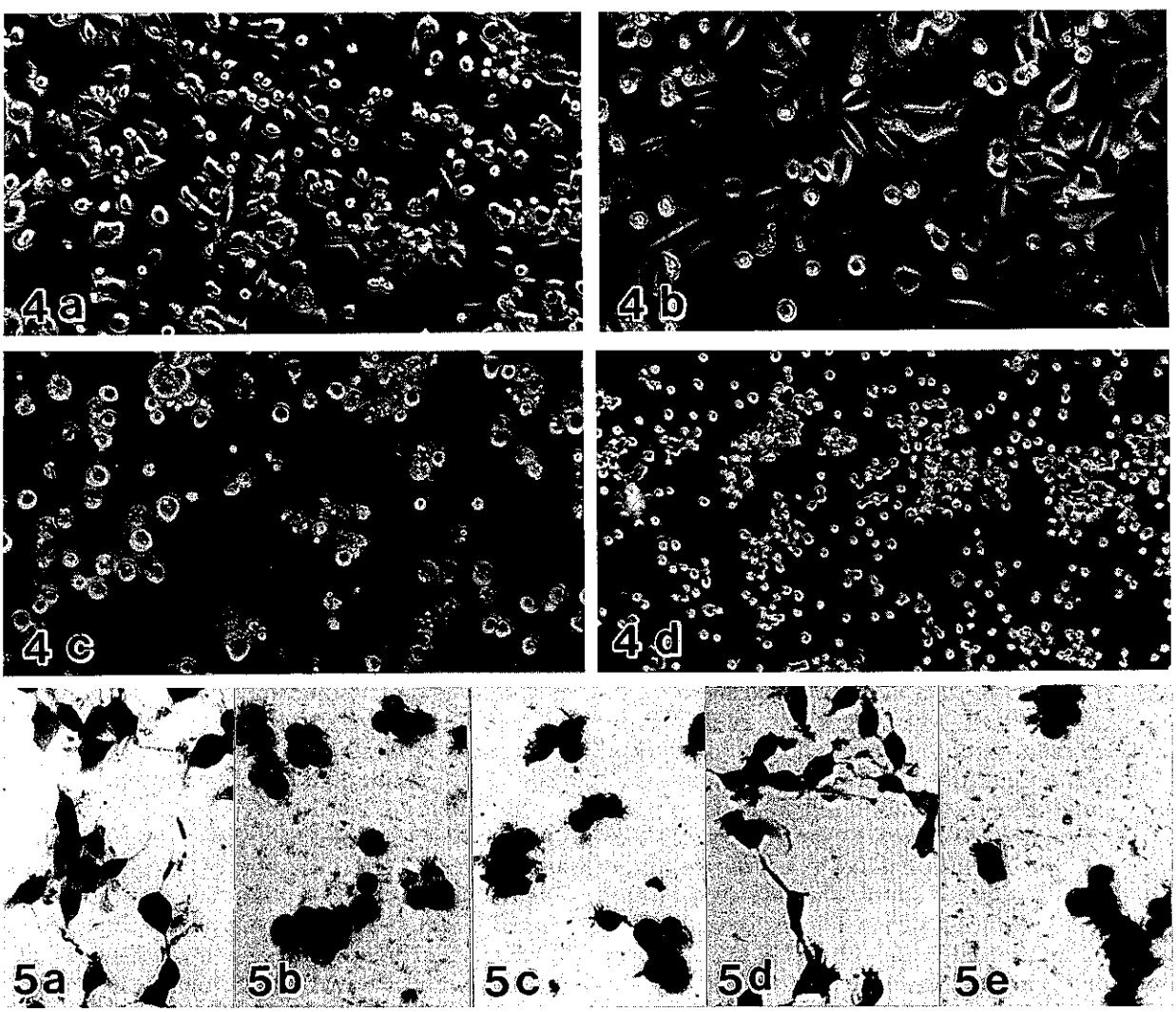

Fig. 4. Cell attachment to plastic culture dish in serum-free medium. A549 lung carcinoma cells (a) and WM115 melanoma cells (b) are spreading after 18 h, while MDA-MB-468 mammary adenocarcinoma cells (c) attach but round-up after 18 h in culture. COLO205 colon carcinoma cells are floating even after 18 h (d). ×140.

Fig. 5. Inhibition of FN-mediated spreading of MCF7 cells by TN. Plastic culture dishes were precoated for 1 h at room temperature with FN, TN, and BSA as follows: a) FN alone; b) TN alone; c) BSA alone; d) FN/BSA (1:1); e) FN/TN (1:1). MCF7 cells were then plated on the dishes, and stained with toluidine blue after 1 h. ×100.

Cell-substrate adhesion Cells cultured for 3 h or 18 h showed differences in shape and attachment to the plastic substrate (Table I) that seemed to correlate with their production of TN and FN (Table II). A549 lung carcinoma cells secreting FN but not TN spread as strongly adherent flat polygonal sheets (Fig. 4a). Cell lines producing both FN and TN behaved variously. Some spread flat such as WM115 (Fig. 4b) and WM266-4 melanomas. Others were round and attached weakly or floated in suspension (Fig. 4c). The latter included cells from five lines (GOTO neuroblastoma, WI38 fetal lung fibroblasts, PA-1 teratocarcinoma, and MDA-MB-468 and MDA-MB-231 mammary adenocarcinomas) that expressed M_r 250,000 TN as well as FN. By contrast, cells secreting both forms of TN and FN spread as well as those that produced only FN. Cells that produced only TN or lacked both FN and TN floated in suspension, even after 18 h of incubation (Fig. 4d). Examples included colon carcinoma, breast carcinoma, leukemia, Burkitt's lymphoma, and epidermoid carcinoma. Lastly, when the ability of TN to inhibit FN-mediated cell spreading was tested, MCF7 cells plated on dishes that were precoated with FN alone or with FN/BSA (1:1) were well spread after a 1 h incubation. However, those cells plated on FN/TN (1:1) or BSA alone remained round and unattached to the substrate (Fig. 5).

DISCUSSION

Among thirty-five human cell lines, five produced FN, four TN, and ten produced both. The remaining sixteen expressed neither. With regard to their attachment behavior in culture, cells that secreted TN alone were round and floated in suspension, like those that produced neither TN nor FN, while cells that secreted only FN attached and spread quickly. Since cells may also release other ECM molecules such as laminin, collagens, and proteoglycans that have been proposed to interact with TN and FN and modulate their cell adhesive functions,[19-22] it is difficult to sort out the specific contributions of each of these molecules to cell adhesion. Nevertheless, the observations suggest that soluble TN released by tumor cells inhibits their binding to FN deposited on the substrate. In a previous study, when fibroblasts from chick, mouse, or rat were plated on FN-coated dishes, the cells attached and spread within 3 h.[19] TN added to the culture medium inhibited attachment in a concentration-dependent manner. Based on this observation, Chiquet-Ehrismann *et al.* postulated that the relative proportions of TN and FN were key determinants of cell shape in culture. In a subsequent study, the spreading and migration of mesodermal cells on FN substrates was found to be inhibited by soluble TN.[23] The present results showing inhibition of MCF7 breast tumor cell spreading on dishes precoated with FN/TN are consistent with this idea. It is also interesting that five cell lines producing soluble M_r 250,000 TN as well as FN were unable to attach to an uncoated plastic substrate. Whether or not TN molecules also manifest this property in the ECM of malignant tumors and their metastases *in vivo* remains a matter of speculation. However, it is conceivable that TN secretion may facilitate subpopulations of cells within a primary tumor to round-up, detach from the substrate and neighboring cells, and invade adjacent normal tissues.

The other findings of this study may be summarized as follows: 1) cell lines derived from both normal epithelial cells and carcinomas synthesized and secreted immunoprecipitable TN *in vitro* in medium containing FCS; 2) normal mammary epithelial (HBL100) and colon carcinoma (COLO205) cell lines secreted TN into serum-free medium, and increased production was induced by TGF-β, but colon carcinoma line COLO320 showed no corresponding increase in TN synthesis above its high baseline level; 3) immunoreactive TN in conditioned medium consisted of two subunits with molecular weights of M_r 250,000 and M_r 190,000. To our knowledge, these results provide the first evidence that TN is synthesized and secreted by several different human carcinoma cell lines as well as by a line derived from normal mammary epithelial cells. Previously, it was thought that mammalian epithelial cells and carcinomas do not produce TN, either *in vivo* or *in vitro*. The fact that TN is secreted by these cells in response to TGF-β[13,24] in serum-free medium offers a new model system for analysis of TN expression by normal and malignant epithelial cells.

A large body of evidence shows that TN is produced by cells derived from mesoderm and neuroectoderm in avian and mammalian species.[1-7] With respect to epithelial cell TN, embryonic chick brain and lung contain TN mRNAs and proteins that are localized in the epithelium of the choroid plexus and bronchial tubules.[25,26] In 1984, Bourdon *et al.* identified a new antigen in human glioma cells and named it glioma-mesenchymal extracellular matrix antigen (GMEM).[27] Using a monoclonal antibody, these authors also examined various human tissues and cell lines by cell surface radioimmunoassay, and they found that normal fibroblasts and cells of neuroectodermal origin as well as malignant sarcomas expressed GMEM. Carcinoma cells and myeloid-lymphoid cells did not. Subsequently, Erickson and Taylor showed that GMEM was biochemically identical with "hexabrachion" proteins in embryonic chick tissues and human tumors.[28] Now known as TN, this protein has received numerous other descriptors including the synonyms "myotendinous antigen,"[3] cytotactin/tenascin,"[1,29-31] "Jl 220/200,"[32] and "hexabrachion."[33]

In the present study, immunoprecipitation with a polyclonal anti-human TN antibody unexpectedly revealed TN secretion by a normal human mammary epithelial cell line and six lines of mammary and colonic adenocarcinomas. The apparent contradiction between these findings and those of Bourdon *et al.* may be related to differences in the sensitivities of the detection methods, immunoprecipitation being more sensitive than cell surface radioimmunoassay. Another explanation may be that the anti-GMEM monoclonal antibody that was used did not recognize an epitope in TN from epithelial cells. It is also possible that the epithelial cell lines tested by Bourdon *et al.* simply did not produce TN under the conditions used. Nevertheless, it is apparent from the current results that both normal and malignant cells of epithelial origin do synthesize TN *in vitro* under serum-free conditions.

During development, TN expression occurs in a complex spatiotemporal sequence beginning at gastrulation.[34] Its decline to the point of disappearance in normal adult cells, including those of epithelial organs, may result from a lack of TN mRNA transcription, due to either the presence of repressor molecules or the absence of transcription initiation factors.[35] When tissues are processed for cell culture, the dissociation of cells releases them from three-dimensional constraints of the surrounding ECM and epithelial-mesenchymal interface relationships. As a consequence, the dynamic balance of gene activity may be tipped in favor of *de novo* synthesis of ECM components for establishing new cell-cell and cell-substrate contacts. In this way, derepression or initiation of TN gene expression in culture may occur when single-cell suspensions are made by proteolysis of tissues *in vitro*.

In conclusion, the basic information provided by this investigation raises a number of interesting questions. Are there subpopulations of malignant cells in some primary carcinomas that express TN or is this a phenomenon confined to the cells in culture? Does TN expression by carcinoma cells *in vivo* increase the probability that they will metastasize? Does TN secretion by metastatic cells make some organs more likely to be invaded than others? Further studies that are needed to address these issues include: 1) correlations between the type(s) of ECM components secreted by different kinds of cancer cells and their specific sites of metastasis; 2) determination of the frequency and location of metastases from TN-producing human carcinomas transplanted into nude mice; 3) measurement of the relative abundance and specific glycosylation patterns of TN isoforms in various normal and neoplastic tissues; and 4) comparison of the patterns of isoform expression in these tissues by *in situ* hybridization and immunohistochemistry. Some of the answers to these questions may lay the groundwork for understanding the functional role of TN in the aberrant malignant behavior of cancer cells.

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