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Production and characterization of monoclonal antibodies specific for different epitopes of human tenascin

Enrica Balza[a], Annalisa Siri[a], Marco Ponassi[a], Fabio Caocci[a], Auli Linnala[b], Ismo Virtanen[b], Luciano Zardi[a,*]

[a]*Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV 10, 16132 Genoa, Italy*
[b]*Department of Anatomy, University of Helsinki, Helsinki, Finland*

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We have obtained and characterized 11 monoclonal antibodies (mAbs) specific for different domains of human tenascin (TN). Five of these mAbs reacted with epitopes contained in the TN area that undergoes alternative splicing and are thus able to recognize specific TN isoforms. These mAbs are a useful tool to study the expression and distribution of TN and its different isoforms in normal and pathological tissues.

Tenascin; Monoclonal antibody

1. INTRODUCTION

Tenascin (TN) is a polymorphic high-molecular-mass extracellular matrix glycoprotein composed of six similar subunits joined together at their aminoterminal by disulfide bonds [1–4]. The sequence of cDNA clones coding for the complete human TN molecule has been reported [5,6]. The deduced amino acid sequence shows that TN is a multidomain protein made up of 14.5 epidermal growth factor (EGF)-like repeats, 15 units similar to fibronectin (FN) type three homology repeats and a C-terminal globular domain similar to β and γ chains of fibrinogen (see Fig. 1). According to a recent paper [7], a fibronectin type three repeat not previously observed is present in human TN between the B and the C repeats in the splicing area.

Recent works have described different TN isoforms generated by alternative splicing pattern of pre-mRNA [8] in which a group of 7 FN-like repeats are included or omitted. Moreover, it has been shown that up to eight different mRNA forms, containing different numbers of FN-like repeats of the splicing area, may be generated by various splicing patterns of pre-mRNA in human cell lines in vitro [6,7]. TN displays a restricted distribution in normal adult tissues and its expression is related to morphogenetic events and embryo development. In fact, TN is transiently expressed in many developing organs and has been proposed as a possible modulator of epithelial–mesenchymal and neuron–glial interactions during organogenesis. Moreover, transient expression of TN is also found in healing wounds and neoexpression or dramatically increased expression has been documented in a variety of tumors [9–15]. MAbs to TN have been employed as in vivo probes for radioisotopic detection and radiotherapy of brain tumors [16–18].

More recently, Oyama et al. [19] and Borsi et al. [20] have demonstrated that in lung cancer and in invasive breast carcinoma the relative amount of the larger TN isoform is higher than in normal tissues. These observations suggest that mAbs reacting with epitopes localized within the splicing area may be useful reagents for clinical applications.

The aim of the present study was to prepare and characterize mAbs specific for different TN isoforms. We have obtained 11 monoclonal antibodies specific for human TN and we have demonstrated that 5 of them react with different epitopes localized in different type III repeats of the splicing area.

2. MATERIALS AND METHODS

2.1. *Cell lines and monoclonal antibodies (mAbs)*

SK-MEL-28 human melanoma and WI38 normal human fibroblast cell lines were purchased from ATCC American Type Culture Collection (Rockville, MD, USA). BHK cells transfected with three different TN cDNA constructs in the pNUT expression vector were a gift of Dr. H.P. Erickson [21]. Two clones, H × B.L and H × B.S produce the large and the small TN splice variants respectively (TN · large, TN · small), and the third (H × B · egf) produces a truncated molecule that terminates after the 14th EGF-like repeat (TN · egf). Human embryonal skin fibroblasts were obtained from local source. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), both obtained from Northumbria Biologicals Ltd. (Cramlington, UK).

The preparation and the partial characterization of the mAbs BC-2, BC-4 and BC-7 have already been reported [6,20]. The mAbs BC-5,

*Corresponding author. Fax: (39) (10) 352-855 *or* 352-999.

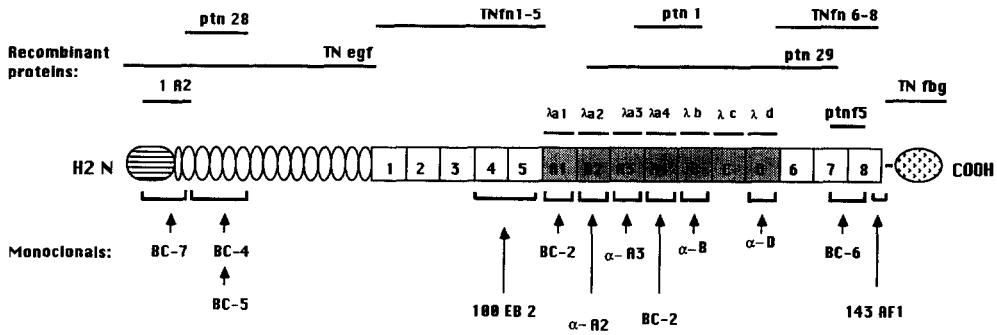

Fig. 1. Model of the domain structure of human TN subunit. The ovals and the squares represent the EGF-like and FN-like repeats, respectively. The globular N-terminal and the fibrinogen-like C-terminal domains are also represented. The FN-like repeats A1 to D whose expression is regulated by the alternative splicing of the pre-mRNA are shaded. The upper part of the figure also shows the TN-β-galactosidase fusion proteins or other recombinant proteins used to localize the epitopes of the mAbs described within the TN molecule. The brackets in the lower part show the shortest sequence in which each epitope was localized.

α-A2 and BC-6 were obtained by immunizing mice as previously reported [22] with TN purified from the conditioned medium of SK-MEL-28 cell line.

The mAbs 100EB2 and 143AF1 were obtained by immunizing mice with TN purified from the conditioned medium of human embryonal fibroblasts and the partial characterization of 100 EB2 has been reported [23].

The mAbs α-A3 and α-D were obtained by immunizing mice with β-galactosidase TN fusion proteins containing the type III repeats A_3 and D, respectively (see Fig. 1), using the expression vector λgt11. Immunofluorescence experiments were carried out as previously described [22]. The isotype of the mAbs was determined with the FITC (fluorescein isothiocyanate) method using a commercial kit purchased from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). Results showed that all the mAbs are IgG_1.

TN was purified from conditioned medium of SK-MEL-28 human melanoma cell line using an immunoadsorbent prepared with the mAb BC-4 as already reported [24]. The same procedure was used to purify the large and small TN splice variants from the conditioned medium of transfected BHK cell lines. The amount of TN was evaluated using the absorption coefficient A_{277} (1 mg/ml, 1 cm) of 0.97 as reported by Erickson and Bourdon [3].

2.2. *cDNA clones and fusion proteins*

All the β-galactosidase–TN fusion proteins reported in this study were obtained using the expression vector λgt11. The clones were obtained either by screening λgt11 cDNA libraries (1 A2; ptn 28; ptn1; ptn 29; ptn f5) or by inserting cDNAs obtained by polymerase chain reaction using appropriate oligonucleotides as primers (λa1; λa2; λa3; λa4; λb; λc; λd). Immunoenzymatic reaction with mAbs was carried out using the ProtoBlot immunoscreening system kit purchased from Promega Biotech (Madison, WI).

PCR reactions were performed for 35 cycles (1 min, 94°C; 1 min, 48°C; 1 min, 68°C) in a final volume of 100 ml containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 mM each dNTP, 100 pmol each oligo, 5 U of Taq DNA polymerase

Table I

Reaction of mAbs in immunoblotting with fusion or recombinant proteins

mAbs	TNegf	1A2	ptn28	TNfn1-5	λa1	λa2	λa3	λa4	λb	λc	λd	ptn29	ptnF5	TNfn6-8	TNfbg	TN · small	TN · large
BC-7	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
BC-4	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
BC-5	±	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
100EB[a]$_2$	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+
BC-2	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+
α-A2	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+
α-A3	−	nd	nd	−	−	−	+	−	−	−	−	−	−	−	−	−	+
α-B	−	nd	nd	−	−	−	−	−	+	−	−	+	−	−	−	−	+
α-D	−	nd	nd	−	−	−	−	−	−	−	+	+	−	−	−	−	+
BC-6	−	nd	nd	nd	−	−	−	−	−	−	−	−	+	+	−	+	+
143AF$_1$	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+
N-end	1	79	<187	620	1071	1162	1253	1344	1435	1526	1617	1244	1803	1708	1973		
C-end	619	>206	333	1070	1161	1252	1343	1434	1525	1616	1707	1890	1911	1972	2199		

Each mAb was tested in immunoblotting with the fusion or recombinant proteins whose sequence was known and with TN large and TN small which represent the two TN isoforms (280 kDa and 190 kDa, respectively) expressed in the transfected BHK cell lines. Results are reported as + or − indicating positive or negative reaction. N-end and C-end represent amino- and carboxy terminal amino acid of each peptide according to the sequence reported by Siri et al. [6]. See also Fig. 1.

[a] Data obtained with fusion proteins TNfn1-3 and TNfn3-5 including the corresponding FN-like type III repeats [21], have shown that this mAb gives a negative reaction with the first and a positive reaction with the second protein (G. Briscoe and H.P. Erickson, personal communication). Thus the epitope recognized by this mAb can be located either in the repeat 4 or in the repeat 5.

(Amplitaq, Perkin Elmer Cetus, Norwalk, CT) and 10 ng of purified DNA as template. All cloning and subcloning procedures were carried out, using the expression vector λgt11 phage, according to Sambrook et al. [25] and each DNA insert was analyzed using Sequenase ver. 2.0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, OH). The β-galactosidase–FN fusion proteins were prepared as previously described [26].

SDS-PAGE and immunoblotting were carried out as previously reported [26].

The clones TN fn1-5, TN fn6-8, TN fbg were a kind gift of Dr. H.P. Erickson [21].

3. RESULTS AND DISCUSSION

We have obtained and characterized 11 mAbs specific for different epitopes of human tenascin. The characterization was obtained through the immunoscreening of cDNA libraries of human cell lines in the expression vector λgt11 and sequence determination of the cDNA of clones expressing positive fusion proteins or by analyzing the reaction of mAbs in immunoblotting with recombinant proteins whose sequence was already known. The results have shown that 5 mAbs recognized epitopes located in the splicing area, while the others reacted with epitopes present in all TN isoforms (Fig. 1, Table I). Since all the mAbs obtained are able to react in immunoblotting, all have been tested with this technique using the available fusion and recombinant proteins (Fig. 2, Table I). This test showed that 10 out of 11 mAbs each reacted with one single domain, while the mAb BC-2 reacted in two different areas of the TN molecule. In fact, testing of this mAb with fusion pro-

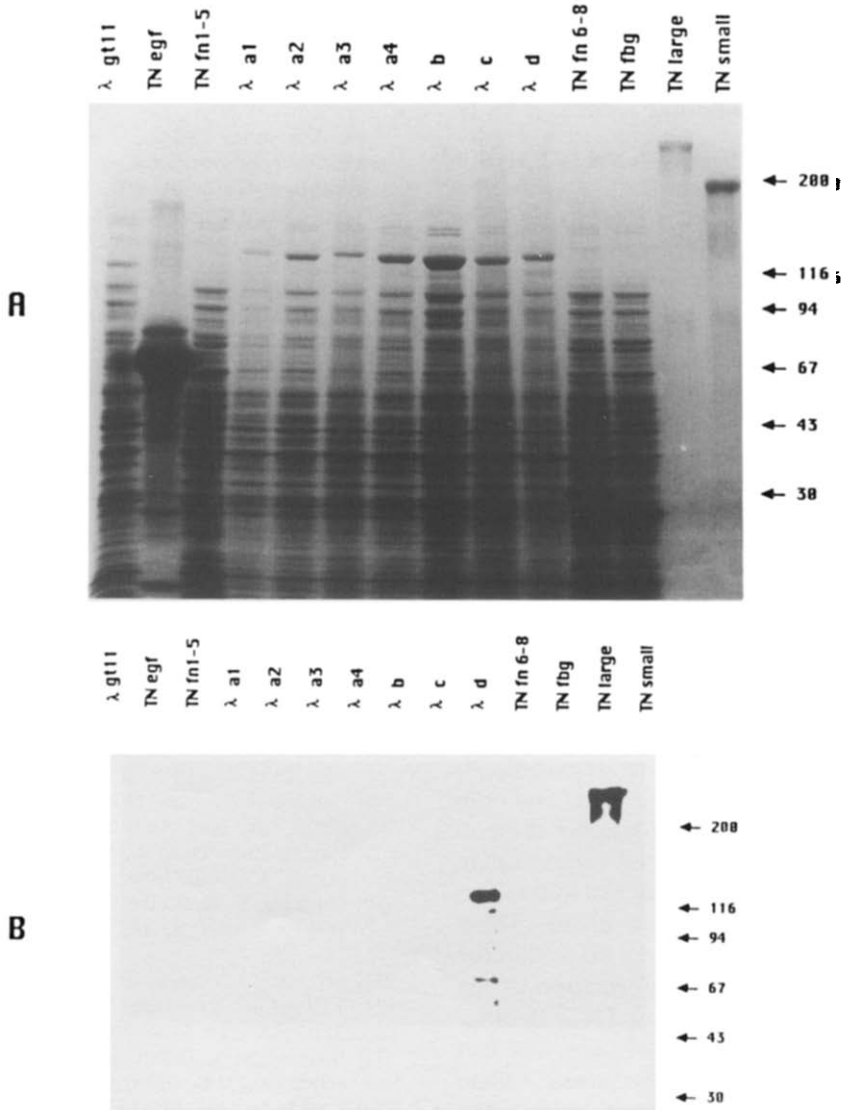

Fig. 2. Reactivity of mAb αD with recombinant and fusion proteins. (A) 4–18% SDS-PAGE gradient of total protein prepared as reported in section 2 from *E. coli* infected by the clone indicated above each lane. TN · large and TN · small are recombinant proteins purified as reported in section 2 and represent the two TN isoforms (280 and 190 kDa, respectively). TN · egf is the supernatant of the BHK cell line releasing the TN form truncated at the end of the 14th EGF-like repeat. (B) Immunoblot of a gel identical to that shown in (A), using the monoclonal α-D. The values on the right indicate the molecular masses in kDa of the standards.

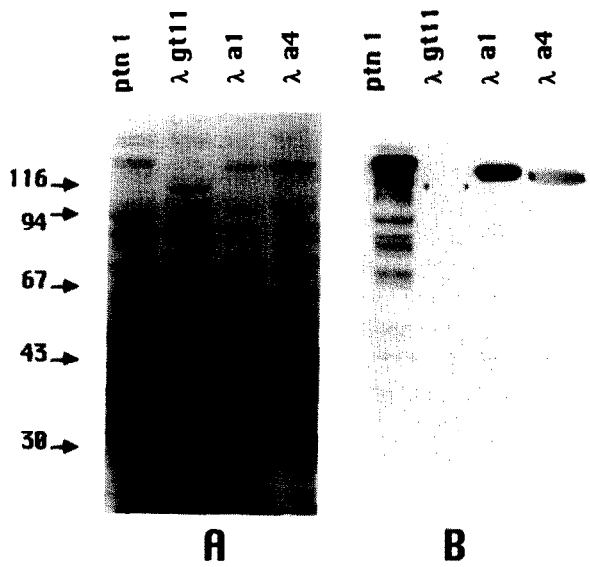

Fig. 3. Localization of the epitopes recognized by the mAb BC-2 using fusion proteins. On the left, a 4–18% SDS-PAGE gradient of total protein from *E. coli* infected with clones containing TN inserts or λgt11 vector as reported above each lane. ptn1 contains the FN-like repeats A4 and nearly a half of each of the repeats A3 and B; λa1 and λa4 contain the repeats A1 and A4, respectively. On the right, immunoblot of an identical gel with the mAb BC-2. The values on the left indicate the molecular masses in kDa of the standards.

teins each containing one single repeat of the splicing area, showed a strong reaction with repeat A1 and a weak reaction with repeat A4 (Fig. 3). This result can be explained by the high percentage of similarity between the two type III repeats A1 and A4 (83%). Moreover, we found a strong reaction with the fusion protein containing repeat A4 and nearly a half of each of the repeats A3 and B: this result suggests that in the case of the epitope contained in the repeat A4, a longer sequence is needed to give rise to an epitope with high affinity for the mAb BC-2.

We have tested the mAbs in immunofluorescence or immunohistochemically on normal human fibroblasts in culture and on several human normal and pathological tissues according to the procedure described. 10 mAbs reacted to different extents in this assay and only one, α-A3, did not show any reaction (data not shown). Furthermore, some mAbs which reacted very strongly in immunoblotting showed a very weak reaction in immunofluorescence experiments (BC-7; BC-6). These data indicate that some epitopes could be masked in the tissues. This could be due to the conformation of the native TN molecule or to interactions of TN with other molecules which could cover some TN regions. The fact that the mAbs αA3, BC-7 and BC-6 reacted well in radioimmunoassay or ELISA experiments using purified native TN (data not shown) reinforce the hypothesis that in tissues some epitopes could be masked because of the interaction with other macromolecules.

Immunohistochemical data show a wide presence of TN in human neoplastic tissues [11,12,14,15]. On the basis of these findings, mAbs specific for TN have enjoyed both diagnostic and therapeutic applications, and successful results have been obtained in glioblastoma treatment [16–18]. It has recently been reported [20] that in invasive breast carcinomas the relative amount of TN mRNA which includes the alternatively spliced region is about 10 times higher than in the RNA from normal breast tissues. Moreover, we have recently observed that the high molecular mass TN isoform in glioma samples represents more than 90% of total TN (Zardi, in preparation), and a similar observation has been reported by Ventimiglia et al. [27] in human glioma cell lines. Given these results, a wider diagnostic and therapeutic use of mAbs specific for the larger TN isoforms might be considered for gliomas or other solid tumors. Moreover, since TN functions and mechanisms that control its expression and splicing pattern are not entirely clarified, these antibodies prove useful tools to contribute to pertinent studies.

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