Tenascin-C Splice Variant Adhesive/anti-Adhesive Effects on Chondrosarcoma Cell Attachment to Fibronectin

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ABSTRACT. Tenascin-C is an oligomeric glycoprotein of the extracellular matrix that has been found to have both adhesive and anti-adhesive properties for cells. Recent elucidation of the two major TNC splice variants (320 kDa and 220 kDa) has shed light on the possibility of varying functions of the molecule based on its splicing pattern. Tenascin-C is prominently expressed in embryogenesis and in pathologic conditions such as tumorogenesis and wound healing. Fibronectin is a prominent adhesive molecule of the extracellular matrix that is often co-localized with tenascin-C in these processes.

We studied the chondrosarcoma cell line JJO12 with enzyme-linked immunoabsorbance assays, cell attachment assays and antibody-blocking assays to determine the adhesive/anti-adhesive properties of the two major tenascin-C splice variants with respect to fibronectin and their effect on chondrosarcoma cell attachment. We found that the small tenascin-C splice variant (220 kDa) binds to fibronectin, whereas the large tenascin-C splice variant (320 kDa) does not. In addition, the small tenascin-C splice variant was found to decrease adhesion for cells when bound to fibronectin, but contributed to adhesion when bound to plastic in fibronectin-coated wells. Antibody blocking experiments confirmed that both the small tenascin-C splice variant and fibronectin contribute to cell adhesion when bound to plastic. The large tenascin-C splice variant did not promote specific cell attachment. We hypothesize that the biologic activity of tenascin-C is dependent on the tissue-specific splicing pattern. The smaller tenascin-C isoform likely plays a structural and adhesive role, whereas the larger isoform, preferentially expressed in malignant tissue, likely plays a role in cell egress and metastasis.

Key words: tenascin-C/splice variant/chondrosarcoma/cell/adhesion/fibronectin

Tenascin-C (TNC) is an oligomeric glycoprotein of the extracellular matrix (ECM) that has been found to be expressed in a variety of processes including cartilage development (Gluhak et al., 1996; Mackie and Murphy, 1998; Mackie and Ramsey, 1996; Pacifici, 1995), tissue remodeling, wound healing (Dalkowski et al., 1999; Hakkinen et al., 2000; Jones et al., 2000; Latijnhouwers et al., 1996), angiogenesis (Gassler et al., 1999; Jallo et al., 1997; Klein-Soyer et al., 1997; Kostianovsky et al., 1997; Nicolo et al., 2000; Ribatti, 1998) and tumorogenesis (Kalembev et al., 1997; Kurpad et al., 1995; Riedl et al., 1997; Vacca et al., 1996; Wilson et al., 1999). TNC has also been implicated in a variety of cell functions including cell adhesion and anti-adhesion, migration and metastasis (Vollmer, 1997). Fibronectin (FN) is another glycoprotein of the ECM that has a more widespread distribution than, but is often co-localized with TNC. Whereas FN is considered an adhesive substrate for cells (Goussia et al., 2000), TNC has been found in some laboratories to prevent cell adhesion to FN (Chiquet-Ehrismann et al., 1988).

Various domains of the TNC molecule have been associated with the adhesive or anti-adhesive properties of the molecule (Angelov et al., 1998; Aukhil et al., 1993; Chung et al., 1996; Elefteriou et al., 1999; Fischer et al., 1997; Fischer et al., 1997; Husmann et al., 1995; Kafitz and Greer., 1998; Kiernan et al., 1996). The molecular structure of TNC is that of a hexabrachion consisting of six subunit ‘arms’. Each arm contains a variable splicing region that contains multiple fibronectin type III (FN-III) repeats (Fig. 1). Alter-
native mRNA splicing creates two major TNC size variants that are identified as 220 and 320 kDa bands on Western blot analysis (Erickson and Bourdon, 1989). In addition, 22 human splice variants have been identified with PCR analysis (Ljubimov et al., 1998). Recent elucidation of the TNC splice variants has shed light on the possibility of multiple functions of the molecule based on the splicing pattern, thus explaining the observation of both adhesive and anti-adhesive properties. (Fischer, et al., 1997)

TNC is expressed in high concentrations in various malignancies including chondrosarcoma, a malignant cartilage-forming tumor (Kalembey et al., 1997; Kurpad et al., 1995; Riedl et al., 1997; Vacca et al., 1996; Wilson et al., 1999). However, TNC is also a major constituent of normal adult articular cartilage (Savarese et al., 1996) and premature cartilage (Pacifici, 1995), suggesting tissue specific function. Preliminary studies in our laboratory have shown a distinctly different in vitro TNC splicing pattern between cultured human chondrocytes and chondrosarcoma cells ((Ghert et al., 2000), submitted for review). The small 220-kDa TNC isoform is the predominant splice variant expressed and produced by the chondrocytes and is incorporated into the matrix. In contrast, the malignant cells express and produce predominantly the large 320-kDa TNC splice variant that exhibits almost no matrix incorporation. The different roles played by the two major TNC splice variants in human articular cartilage and chondrosarcoma cells may be a function of the adhesive/anti-adhesive properties of each splice variant. Elements of the metastatic cascade include cell detachment from the matrix and cell egress, both requiring anti-adhesive matrix components. We have used enzyme-linked immunoabsorbance assays, cell attachment assays and antibody blocking assays to determine the specific adhesive/anti-adhesive properties of the two major TNC splice variants with respect to chondrosarcoma cell attachment to FN. We hypothesize a possible role for the specific TNC splicing pattern in the metastatic behavior of the chondrosarcoma cells.

Materials and Methods

Enzyme-linked immunoabsorbance assay

Purified human FN (Chondrex, Seattle, WA, USA) was diluted in phosphate buffer saline (PBS) to a concentration of 2 µg/ml. Human TNC large and small expression proteins (HxB.L and HxB.S) corresponding to the 320 kDa and 220 kDa proteins were diluted to a concentration of 10 µg/ml. Human TNC was purified from conditioned medium of U-251MG human glioma cells by gel filtration and Mono Q chromatography (Aukhil et al., 1990; 1993) (H.Erickson, Duke University, Durham, NC, U.S.A.). The chosen concentrations (TNC five times less adhesive than FN) were based on pilot cell adhesion assays to determine the optimal cell binding concentration for each matrix protein (data not shown). Ninety-six-well immunoabsorbent plates (Maxi Sorp; Nunc, Denmark) were coated overnight at 4°C with 50 ml of the test protein (TNC or FN for single-protein wells and FN for double-protein wells). All wells were then rinsed with PBS.

In experiment A, the wells were blocked for 1 hour at 37°C with 1% bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO, USA) following FN adsorption and prior to the second protein coat (Fig. 2). In this manner, the plastic spaces left open by the FN coat were blocked by the BSA and nonspecific plastic binding sites were not available for the second protein coat. In experiment B, the second protein coat (TNC large or small protein) was added overnight at 4°C prior to BSA blocking (Fig. 3). Therefore, in experiment A the TNC could bind only to the FN and not to the plastic, and in experiment B the TNC either could bind to the FN in or to the plastic exposed in the pores in between FN molecules. Negative control wells consisted of BSA coating only and were used to

TNC:

Fig. 1. Schematic diagram of the TNC hexabrachion arm. The tenascin assembly (TA) links six TNC chains via the heptad repeats. Proceeding in a carboxyl terminal direction, the domains are as follows: an array of 14.5 epidermal growth factor like repeats (EGFL), two types of FN-III domains: (1) those conserved in all variants of TNC (white boxes), and (2) those that are alternatively spliced (gray boxes), and the terminal fibrinogen knob (FG globe). In TNC 320, all alternatively spliced segments are present and in TNC 220, none of the alternatively spliced segments are present.
subtract background adhesion.

The wells were rinsed with PBS and incubated for 2 hours at 37°C in a 1/2000 dilution of the rabbit polyclonal anti-TNC antibody HxB.9504 (produced against FN-III domains 6-8 and the C-terminal knob, which is identical for both TNC splice variants, see Figure 1) (H.Erickson, Duke University, Durham, NC, U.S.A.) (Spring et al., 1989). The wells were rinsed with PBS-Tween (PBS-T) and incubated for 1 hour at 37°C with a secondary anti-rabbit IgG horseradish peroxide conjugated antibody (ECL, Amersham Pharmacia, Biotech, England). A peroxide-reactive color developing system and absorbance at 490 nm was used to quantitate the amount of protein bound.

**Cell adhesion assay**

Ninety-six-well immunoplates (Maxi Sorp; Nunc Denmark) were prepared as above for the ELISA protocol. The chondrosarcoma cell line, JJ012, was provided by Dr. Joel Block (J. Block, Rush-Presbyterian St. Luke’s Medical Center, Chicago, IL, U.S.A.). The cells were cultured in a monolayer in basal media supplemented with 10% fetal bovine serum (FBS), split and re-plated at half-confluence two days prior to the assay. The cells were released from the substratum with 1mg/ml trypsin, washed several times in 0.5% soybean trypsin inhibitor (Sigma Chemical, St. Louis, MO) and diluted in media to a concentration of 1x10^6 cells/ml. At total of 1x10^5 cells (100 μl) were added to each well and incubated for 1 hour at 37°C. The wells were rinsed with PBS and a hexosaminidase substrate was added to the wells and incubated for 3 hours at 37°C. The cell binding was quantitated by measuring endogenous hexosaminidase as previously described (Landegren, 1984).

**Antibody-blocking cell adhesion assay**

The cells adhesion assays were performed as described above. However, prior to the addition of the JJ cells to the prepared plates, all wells were incubated for 2 hours at 37°C in 100 μl of one of three antibody blocking solutions. These solutions were: (1) a 1/500 solution of an antibody directed against the C-terminus of TNC (HxB.9504) (2) a 1/50 dilution of a mouse monoclonal antibody to the cell-binding site of FN (HB5) (H.Erickson, Duke University, Durham, NC, U.S.A.) (Spring et al., 1989).
University, Durham, NC, U.S.A.). (Spring, 1989), or (3) a 1:1 combined solution of TNC (HxB.9504) and FN (HB5) antibodies at the above dilutions. Following the antibody-blocking step, the wells were rinsed twice with T-PBS and twice with PBS to eliminate unbound antibodies. The JJ cells were then added to the wells as described above and attachment assays were performed.

Data analysis

All experiments were performed in triplicate. Data analysis was performed using t-test comparison of means (Microsoft Excel). A P-value of less than 0.05 was considered statistically significant.

Results

Enzyme-linked immunoabsorbence assay

In experiment A in which BSA in between protein coats prevented nonspecific TNC binding to plastic, the small 220 kDa TNC splice variant bound to FN to a significantly greater extent than did the 320 kDa TNC splice variant (P<0.001) (Fig. 4). The large TNC splice variant had negligible binding to FN. In contrast, in experiment B in which the BSA was added after both protein coats and nonspecific TNC binding to plastic was possible, both the small and large TNC splice variants bound to the FN-coated wells (Fig. 4). Of note, ELISA detection of FN in this experiment showed that the FN concentration did not decrease with the second protein coat nor between experiments A and B (data not shown). These results indicate that the small TNC isoform binds to FN and that the large TNC isoform does not. However, if plastic sites are available for nonspecific binding in the FN-coated wells, the large TNC splice variant will bind in the wells.

Cell attachment assay

In experiment A, JJ cell attachment to FN was unaffected in the wells where the large TNC 320 kDa protein was added as a second coat (P=0.15) (Fig. 5). This negative result is consistent with the absence of binding of TNC 320 in this protocol. In contrast, cell binding to FN was significantly inhibited in the wells where the small 220-kDa protein was bound to FN (P<0.01). These results indicate that the specific binding of the small TNC isoform to FN inhibits chondrosarcoma attachment to FN in vitro. In contrast, in experiment B where BSA was added after both coats, the attachment of the small TNC protein to the available plastic sites significantly increased JJ cell adhesion to the FN-coated wells (P<0.05) (Fig. 5). The mechanism through which this increased adhesion occurs was not further explored in this study. The attachment of the large TNC protein to the plastic sites did not affect JJ cell adhesion to the FN-coated substrate (P=0.15). This suggests that the small 220-kDa TNC splice variant has adhesive properties for chondrosarcoma cells if the protein is allowed first to bind

Experiment A: BSA in between protein coats

![Graph showing quantitation of bound substrate in enzyme-linked immunoabsorbence assay with the polyclonal antibody HxB.9504 (anti-TNC terminal knob, identical for all TNC). All experiments were performed in triplicate. The error bars represent standard deviation and statistical analysis was performed with the use of the student’s t-test analysis of variance. A P-value of <0.05 was considered statistically significant. In experiment A (top) in which nonspecific binding sites were blocked after coating with FN, significantly more of the small TNC splice variant bound to FN than did the large TNC splice variant (**P<0.001). The large TNC isoform had negligible binding in the wells. In experiment B (bottom) in which the TNC protein layer was exposed to both FN and plastic binding sites, both the large and small TNC splice variants were found to bind to FN (**P<0.01).

Experiment B: BSA after both protein coats

![Graph showing quantitation of bound substrate in enzyme-linked immunoabsorbence assay with the polyclonal antibody HxB.9504 (anti-TNC terminal knob, identical for all TNC). All experiments were performed in triplicate. The error bars represent standard deviation and statistical analysis was performed with the use of the student’s t-test analysis of variance. A P-value of <0.05 was considered statistically significant. In experiment A (top) in which nonspecific binding sites were blocked after coating with FN, significantly more of the small TNC splice variant bound to FN than did the large TNC splice variant (**P<0.001). The large TNC isoform had negligible binding in the wells. In experiment B (bottom) in which the TNC protein layer was exposed to both FN and plastic binding sites, both the large and small TNC splice variants were found to bind to FN (**P<0.01).
to plastic. If the small TNC isoform is bound to FN, the double-protein complex has anti-adhesive properties (Fig. 5).

**Antibody blocking attachment assays**

As displayed in Figure 6, experiment A, the blocking of the TNC with the polyclonal antibody HxB.9504 was performed after both protein coats (BSA in between coats). This was examined for TNC 220 and FN combinations only, as TNC 320 was found in the cell adhesion assays above not to have any significant effect on cell adhesion. In all wells, the antibody did not significantly affect JJ cell attachment. Of note, the results of cell attachment assay A were confirmed: the TNC220:FN complex significantly inhibited JJ cell attachment ($P<0.05$). We hypothesize that the reason that the anti-TNC antibody did not affect cell adhesion in the TNC220:FN wells is because the cell-binding sites may already be occupied in the TNC220:FN binding complex.

When BSA blocking was performed after the two protein coats (both FN and TNC220 were bound to plastic and available for cell attachment) (experiment B), the addition of either anti-FN (HB5) or anti-TNC (HxB.9504) antibody decreased cell binding (Fig. 7). However, the greatest decrease in cell binding was found in the wells with both antibodies ($P<0.05$). These results indicate that both proteins are responsible for cell binding. Of note, the cell binding in the wells with both TNC220 and FN was increased compared to wells with FN alone ($P=0.05$), verifying the results shown in Figure 5.

**Discussion**

In this study we found that at maximal cell-binding concentrations, the small TNC isoform (220 kDa) binds to FN whereas the large TNC isoform (320 kDa) does not. Both proteins bind to plastic. However, the TNC220:FN complex is less adhesive for chondrosarcoma cells than is FN alone. If, however, the small TNC splice variant binds to plastic, it has additive adhesive properties with FN. The large TNC splice variant did not have adhesive properties in any of the assays performed. The cell-binding abilities of the TNC splice variants alone are significantly less than that of FN alone, implicating FN as a major adhesive molecule of the ECM.

Earlier studies in the literature reported that TNC inhibits cell attachment to FN (Chiquet-Ehrisman et al., 1988; Lightner, 1990; Lotz et al., 1989). One mechanism for the inhibition was steric blocking, in which the large hexabrachion covered up the adhesion sites on FN when it was able to be bound to unblocked plastic (Joshi et al., 1993; Lightner, 1990). Other mechanisms involved possible interactions of specific tenasin segments with cell receptors (Lotz et al., 1989; Yokosaki, 1998). It is possible that the adhesive/anti-adhesive role played by TNC is determined by its tissue-specific splicing pattern. The more recent elucidation of the various splice variants and techniques to reconstruct them have led to more detailed investigation of the adhesive/anti-adhesive properties of the two major isoforms (220 kDa and 320 kDa) (Spring et al., 1989).

In support of our results, several investigators have found
that FN binds preferentially to the smaller TNC isoform. Chiquet-Ehrisman et al. used affinity chromatography and a solid-phase binding assay to study the interaction of FN with the two major chicken TNC isoforms (Chiquet-Ehrisman et al., 1991). The authors demonstrated that the small variant bound preferentially to fibronectin in enzyme-linked immunosorbent assay, and only the small variant was incorporated into the matrix by cultures of chicken fibroblasts. Chung et al. studied human TNC, and confirmed that the small variant binds preferentially to purified fibronectin and to fibronectin-containing extracellular matrix (Chung et al., 1995). Using bacterial expression proteins, the authors mapped the major binding site to the third FN type III (FN3) region and to a minor binding site in the FN6-8 domain which is only positioned to bind fibronectin in the small splice variant.

Various other studies have verified that the small TNC isoform is concentrated in areas of cartilage deposition and matrix incorporation and have suggested that this phenomenon may be due to the small TNC protein interaction with FN (Chiquet-Ehrisman et al., 1991; Carnemolla et al., 1992; Chiquet-Ehrisman, 1993). The same investigators found that the larger TNC isoform does not accumulate at the site of synthesis but instead diffuses out of the matrix and accumulates in the media.

We found that the small TNC protein is adhesive for chondrosarcoma cells when bound to plastic in a mixture with FN. However, when bound to FN molecules, the TNC220:FN binding complex inhibited cell attachment. Although we are the first, to our knowledge to find that the small TNC splice variant alone has additive adhesive properties with FN, others have found that the TNC220:FN binding complex is anti-adhesive. Hauzenberger et al. used attachment assays, enzyme-linked immunoabsorbance assays and TNC molecules of various sizes to determine the effect of TNC on T-lymphocyte adhesion to fibronectin (Hauzenberger et al., 1999). The authors demonstrated that the TNC FN type III domains 1-5 bound to fibronectin and that the variable splicing region did not. In addition, the binding of the small TNC fragment inhibited antibody detection of the TNC binding site and inhibited T-lymphocyte attachment to FN. The authors concluded that the inhibition of cell attachment to FN involves the binding of TNC repeats TNfnIII 1-5 to FN.

Chung et al. likewise mapped the major FN binding site on TNC to the FNIII-3 domain and found that a monoclonal antibody against an epitope in this domain did not stain TNC segments bound to cell culture matrix fibrils (Chung et al., 1995). Fischer et al. used mutant TNC proteins to determine the cell-attachment roles of the various molecular domains (Fischer et al., 1997). The authors found that the fibrinogen knob was adhesive for cells. However, when TNC was added to the medium of fibroblasts plated on fibronectin-coated wells, cell adhesion was blocked by the interaction of the fibrinogen domain with FN. Similarly, Aukhil et al. mapped a cell-binding site on TNC to the fibrinogen domain. A growing body of literature therefore suggests that the small TNC isoform is able to bind FN at two possible binding sites, and that this binding blocks cell adhesion to both TNC 220 and FN. Conversely, the small
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TNC protein that is bound to plastic has both binding sites exposed and has adhesive properties for cells.

A finding in this study that contradicts previous investigations is that the large TNC splice variant, when bound to plastic, did not block cell adhesion to FN. These are in contrast to the findings of Lightner et al. who demonstrated that when bound to plastic, the large TNC molecule blocked cell adhesion to FN (Lightner, 1990). The authors suggested the possibility of a steric blocking mechanism for this observation. It is possible that the discrepancy in these results is related to the protein coat preparation. In the protocol published by Lightner et al., the protein coats were incubated at room temperature for 30 minutes. In our protocol, we incubated the protein overnight at 4°C. We hypothesize that a conformational change occurs in the longer, colder incubation period in which the large TNC molecule loses its steric blocking three-dimensional structure. Another possible explanation for the discrepancy in results is the difference in cell lines studied with theoretically different matrix-binding properties. Further study is needed to clarify this phenomenon.

We have previously determined in our laboratory that normal human chondrocytes, when cultured in an alginate bead system, produce a large quantity of the small 220 kDa TNC protein and incorporate it into the matrix ((Ghert et al., 2000), submitted for review). Chondrosarcoma cells have a distinctly different TNC splicing pattern than the chondrocytes in vitro. These cells produce predominantly the large 320-kDa-splice variant, which is not incorporated into the matrix but is diffused into the media. These latter results are supported in the literature for several other malignancies (Borsi et al., 1992; Carnemolla et al., 1992; Wilson et al., 1996). We have continued our investigation in order to clarify the role that the two major TNC isoforms play in the adhesive/anti-adhesive properties of the chondrosarcoma cell. We found that the 320-kDa TNC protein does not bind to FN and does not promote cell adhesion. The 220 kDa TNC protein, however, was found to bind to FN and to have both adhesive and anti-adhesive properties, depending on the substrate to which it is bound.

The metastatic cascade involves several steps including cell-matrix detachment, cell migration and invasion. The activity of the 320 kDa TNC protein in tumor matrix is likely related to the induction of matrix degradation, invasion and cell migration (Jähkola et al., 1996, 1998; Shrestha et al., 1996; Tremble et al., 1994; Yoshida et al., 1999). These processes may involve functions other than cell-matrix anti-adhesion, namely the induction of proteolytic enzymes such as the metalloproteinases and basement membrane invasion. These other functions of TNC320 in malignant tissue would explain the lack of significant cell-matrix anti-adhesion found for TNC320 in this study. Further investigation into the possible roles for TNC320 in the process of metastasis is warranted.

The presence of the small TNC splice variant in the matrix of normal cartilage and the adhesive properties demon-
strated in this study indicate a role for matrix-matrix and cell-matrix adhesion. The biologic activity of TNC in various tissues is therefore dependent on the tissue specific splicing patterns. The smaller TNC isoform likely plays a structural and adhesive role, whereas the larger isoform likely plays a role in tissue breakdown, cell migration and metastasis. Further elucidation of these specific roles has the potential to lead to novel therapeutic approaches to chondrosarcoma and other malignancies.

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References


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