Tenascin-C induced stimulation of chondrogenesis is dependent on the presence of the C-terminal fibrinogen-like globular domain

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Abstract The relationship between structure of tenascin-C (Tn-C), a multi-domain extracellular matrix protein, and its stimulation of chondrogenesis was examined using recombinant Tn-C isoforms (full length or with specific domains deleted) as substrata for undifferentiated chicken mesenchymal cells. Of the Tn-C variants tested, only Tn-C lacking the fibrinogen-like domain or Tn-C comprised solely of fibrinogen-like domains failed to stimulate chondrogenesis. The ability of variants to stimulate chondrogenesis was not dependent on their ability to support adhesion or stimulate proliferation. These results demonstrate that the fibrinogen-like domain of Tn-C is necessary but not sufficient for induction of chondrogenesis.

Key words: Tenascin-C; Chondrogenesis; Chick embryo wing bud cell

1. Introduction

Development of a chondrogenic cell lineage from undifferentiated mesenchymal cells in the limb bud is an important process in endochondral bone formation. Chondrogenic events include: mesenchymal cell rounding and aggregation of resultant chondroblasts; proliferation and maturation of aggregated chondroblasts with expression of cartilage specific proteins, such as type II collagen, proteoglycans and hyaluronan [1–3] to form chondrogenic nodules; and hypertrophy of mature chondrocytes with expression of type X collagen.

The extracellular matrix (ECM) is an important regulator of chondrogenesis and separation of chondrocytes from their ECM induces de-differentiation [4]. The ECM glycoprotein tenascin-C (Tn-C) appears to play a role in early differentiation events. Tn-C is present in condensing mesenchyme of embryonic cartilages, however, it is lost from cartilage with progressive chondrocyte maturation [5,6]. In vitro, Tn-C stimulates chondrogenesis in cultures of mesenchymal cells from chicken embryo wing buds (CEWB) [5] and antibodies against Tn-C inhibit chondrogenesis [7].

Tn-C is a hexamer, formed by disulfide bonding between N-terminal domains of two trimers. Monomers (see Fig. 1) comprise fibronectin (Fn) type III-like and epidermal growth factor (EGF)-like repeated domains and a single β/γ fibrinogen-like domain [8]. Chicken Tn-C has eight constitutive Fn type III-like repeats, six non-constitutive Fn type III-like repeats (A, B, D, AD2, AD1 and C) produced as a result of alternative splicing and 13.5 EGF-like repeats [9].

Structure–function relationships of Tn-C domains were investigated with respect to stimulation of chondrogenesis in CEWB cells cultured on substrata of recombinant Tn-C. A number of full length recombinant Tn-C isoforms and variants of the smallest of these, Tn-190, with specific domains deleted were used. Electron microscopy of these recombinant Tn-C variants showed them to be hexamers and to be folded as native Tn-C [10]. Chondrogenic nodule formation was used as a marker of chondrogenesis.

2. Materials and methods

2.1. Recombinant Tn-C production

Production of recombinant Tn-C has been described in detail elsewhere [10,11]. Essentially, variants were prepared from full length cDNA for the 230 kDa species of chicken Tn-C using a PCR based method, splicing by overlap extension [12]. Constructs were subcloned into the eukaryotic expression vector pCNAI/NEO and expressed in HT1080 human fibrosarcoma cells. Tn-C variants were purified from the conditioned culture medium using immunoaffinity chromatography with monoclonal antibodies appropriate for specific variants [10].

2.2. Primary culture of CEWB cells

CEWB cells were isolated from stage 23–24 [13] White Leghorn chicken embryos with 0.1% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ, USA) in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (10 min, 37°C with shaking). Cells were washed with Dulbecco's minimal essential medium containing 10% fetal calf serum (FCS), gentamicin (50 μg/ml) and amphotericin B (2.8 μg/ml; DMEM) and filtered through 40 μm nylon mesh. Final cell density was adjusted to 8×10^5 cells/ml with DMEM.

2.3. Chondrogenesis assay

For preparation of substrata, individual wells of 96 well tissue culture dishes were incubated with 50 μl/well PBS or recombinant Tn-C (50 μg/ml) in PBS for 1 h at room temperature (RT). 50 μl DMEM was added to each well followed by 50 μl CEWB cell suspension. Final cell density was 4×10^4 cells/well. Cells were cultured (37°C, 5% CO_2) for 3–4 days with DMEM replaced daily. In some experiments, numbers of non-attached cells were counted after 18 h in culture. Cells were fixed (4% paraformaldehyde in PBS, overnight, 4°C) and highly sulfated glycosaminoglycans in the chondrogenic nodules were stained using 0.5% Alcian blue dye in 3% acetic acid, pH 1.2 (1 h, RT). Following 3% acetic acid destain washes, Alcian blue stained nodules were counted.

2.4. Cell attachment assay

Individual wells of a 96 well tissue culture dish were incubated with PBS, recombinant Tn-C or fibronectin (20 nM) to form substrata, as described. Wells were then incubated with 1% heat denatured (80°C, 15 min) bovine serum albumin for 1–2 h. CEWB cells were prepared

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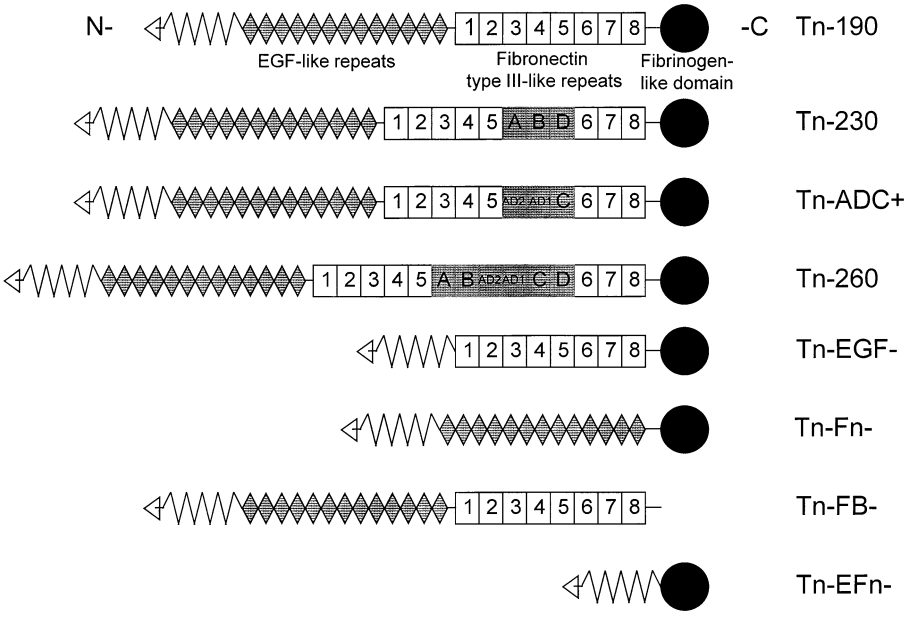

Fig. 1. Representation of monomer units of recombinant Tn-C hexamers used in the current study. Non-constitutive Fn type III-like repeats are filled. Tn-190, Tn-260, Tn-230 and Tn-ADC+ represent splice variants known to occur in vivo.

as described above and were cultured overnight. CEWB cells were incubated with 0.05% trypsin in Ca^{2+} and Mg^{2+} free PBS (10 min, 37°C) and non-attached cells were washed with DMEM in the absence of FCS. Cells were added to the prepared substrata at 4×10^4 cells/well in 0.1 ml and incubated at 37°C in 5% CO_2 for 90 min. Non-adherent cells were removed with the medium and adherent cells were fixed with 4% paraformaldehyde and stained with 1% toluidine blue (2 h, RT). After destain washes with PBS, cells were lysed with 2% sodium dodecyl sulfate and optical density at 600 nm was measured.

2.5. Cell proliferation assay

CEWB cells (4×10^4 cells/well) were cultured on substrata of recombinant Tn-C or on tissue culture plastic only. After 24 h, fresh DMEM containing 10 μCi/ml [^{3}H]5-methylthymidine (Amersham Life Sciences, Amersham, UK) was added. Cells were incubated for a further 18 h before being dislodged by trypsinization (0.05% trypsin, 10 min, 37°C), transferred to 96 well dishes with filter bases (Multiscreen™ Assay System, Millipore, Watford, Hertfordshire, UK) and washed using vacuum filtration. DNA was precipitated with 20% trichloroacetic acid (TCA; overnight, 4°C) and washed with 10% ice-cold TCA. Filters were transferred to scintillation vials containing 0.4 ml of a 0.42% (v/v) household bleach solution and were incubated overnight at RT. Scintillation fluid was added (4 ml/vial) and incorporated [^{3}H]thymidine was measured using a scintillation counter.

2.6. Statistical analysis

Data were analyzed by Student's *t*-test or analysis of variance with multi-variant analysis using SPSS computer software.

3. Results

3.1. Effect of recombinant Tn-C substrata on chondrogenesis

With the exception of two variants, culturing undifferentiated CEWB cells on a substratum of recombinant Tn-C increased numbers of Alcian blue stained chondrogenic nodules significantly ($P<0.05$; Figs. 2 and 3). No significant difference in chondrogenic nodule count was recorded for CEWB cells cultured on tissue culture plastic (control) and those cultured on Tn-FB−, which lacks the fibrinogen-like domain. Tn-230, a full length Tn-C including non-constitutive Fn type III-like repeats A, B and D, and Tn-260 containing all six non-constitutive Fn type III-like repeats, elicited a five-fold increase in chondrogenic nodule number compared with control. The highest increase (≥10-fold) was achieved with Tn-190, the shortest full length recombinant Tn-C (containing only constitutive Fn type III-like repeats), Tn-Fn− (lacking all Fn type III-like repeats), Tn-EGF− (lacking all EGF-like repeats) and Tn-ADC+ (a full length isomer including non-constitutive Fn type III-like repeats AD2, AD1 and C).

Tn-EFn− lacks EGF- and Fn type III-like repeats and is essentially a hexamer of fibrinogen-like domains only. Culturing CEWB cells on a Tn-EFn− substratum failed to stimulate chondrogenesis (Fig. 3).

3.2. Effect of recombinant Tn-C substrata on CEWB cell adhesion and proliferation

Chondrogenic nodule formation in CEWB cell cultures is dependent partly on cell density [14] which, over a 3 day

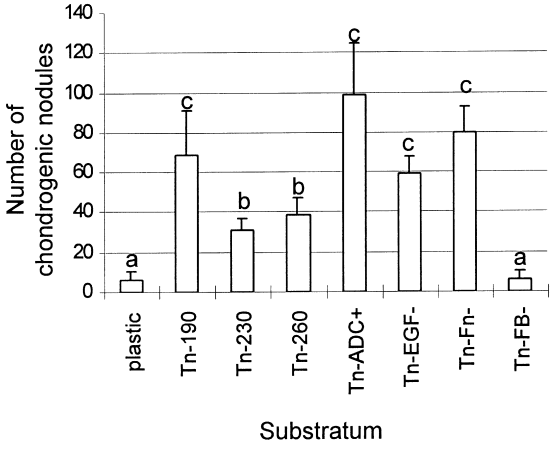

Fig. 2. Chondrogenic nodule formation in CEWB cells cultured on Tn-C variants. Mean ± S.E.M., $n=8$. Analysis of variance defined three groups with significantly different mean Alcian blue stained chondrogenic nodule values (a, b and c) using 95% confidence limits.

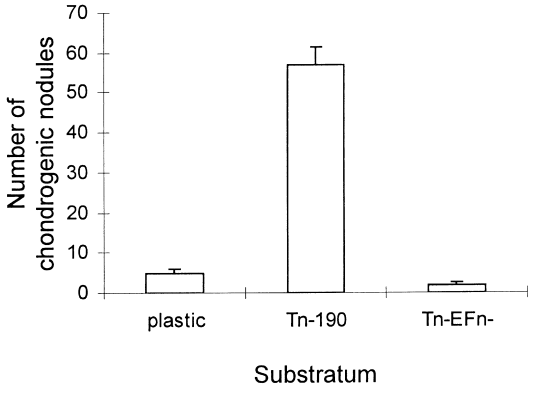

Fig. 3. Chondrogenesis in CEWB cells cultured on tissue culture plastic, Tn-190 or Tn-EFn. Mean ± S.E.M. of three experiments.

culture period, is dependent on cell adhesion and proliferation. Adhesion assays showed that in the absence of serum, tissue culture plastic, Tn-190, Tn-230, Tn-ADC+ and Tn-FB− allowed attachment of approximately half as many cells as fibronectin (Fig. 4). Attachment of cells to Tn-260 was reduced compared with other Tn-C substrata ($P < 0.05$), whereas Tn-EFn− showed enhanced attachment ($P < 0.05$). Cell proliferation was also investigated (Fig. 5). Most substrata had no effect on [^{3}H]thymidine incorporation compared with cells cultured on tissue culture plastic alone. Tn-190 and Tn-ADC+, however, significantly increased [^{3}H]thymidine incorporation above control ($P < 0.05$). Data are expressed as [^{3}H]thymidine incorporation/well and do not take into account initial cell number, which is influenced by cell adhesion. [^{3}H]Thymidine incorporation per cell for Tn-190 and Tn-ADC+ was calculated using 18 h cell numbers to be 187 and 195% of control, respectively.

4. Discussion

The majority of recombinant Tn-C variants tested, including the shortest full length recombinant Tn-C, Tn-190, stimulated chondrogenesis in CEWB cell cultures. Of the larger variants, only that lacking the fibrinogen-like domain (Tn-FB−) was unable to stimulate chondrogenesis indicating that this domain is critical for Tn-C activity. A variant consisting only of a hexamer of fibrinogen-like domains was, however, unable to stimulate chondrogenesis, indicating that the spatial arrangement of the domains is probably important. Since both Tn-EGF− and Tn-Fn− stimulated chondrogenesis, it appears that EGF-like repeats and Fn type III-like repeats are interchangeable as spacers. Inclusion of non-constitutive Fn type III-like repeats A, B and D in Tn-230 decreased chondrogenic potential. This may help to explain the distribution of Tn-C isoforms in the developing chicken long bone, as reviewed in [6]; Tn-190 is the major isoform expressed by cells actively synthesizing cartilage, whereas osteogenic cells, which are not involved in cartilage production, express Tn-230. Undifferentiated mesenchymal cells express both isoforms, since cell lineage has yet to be determined. Tn-ADC+, which includes the non-constitutive Fn type III-like repeats AD2, AD1 and C, stimulated chondrogenesis at or above Tn-190 levels, suggesting that the partial inhibition seen with Tn-230 is specific for Fn type III-like repeats A, B and D and is not caused by interruption of constitutive Fn type III-like repeats 5 and 6.

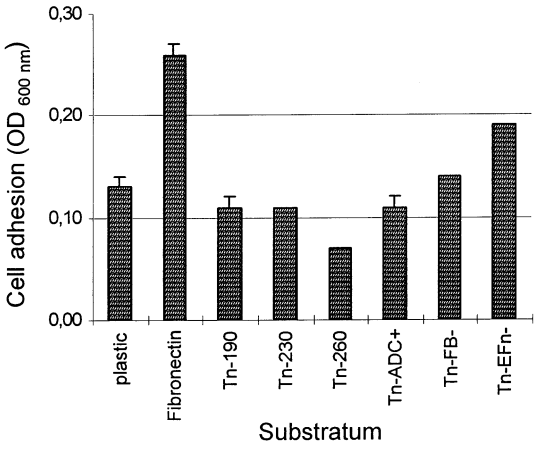

Fig. 4. Attachment of CEWB cells to Tn-C variants or fibronectin. Mean ± S.E.M., $n = 6$.

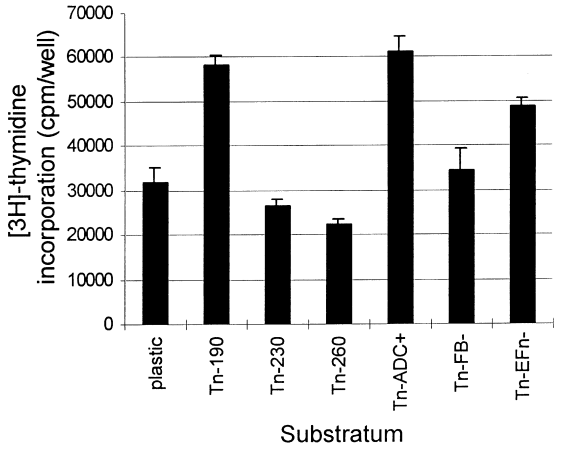

Fig. 5. Incorporation of [^{3}H]thymidine into CEWB cells cultured on Tn-C variants. Mean ± S.E.M., $n = 6$.

In mesenchymal cell cultures, only cells at high density will undergo chondrogenic differentiation. It was, therefore, necessary to determine whether differences in chondrogenesis stimulating activity between variants could be attributed to differences in adhesive or mitogenic properties. Indeed, the fibrinogen-like domain, which was critical for chondrogenesis, has been shown to be important for binding of Tn-C to smooth muscle cells [15] and fibroblasts [10,16]. Since the aim of these experiments was to determine whether cell–substratum adhesion affected the outcome of chondrogenesis assays, adhesion assays were carried out in the same type of tissue culture plastic as the chondrogenesis assay. Therefore, unlike controls for adhesion assays carried out on bacterial plastics, in this study, control wells provided a moderately good substratum for adhesion, although not as good as fibronectin. In comparison with tissue culture plastic, only Tn-EFn− promoted cell adhesion while Tn-260 demonstrated anti-adhesive activity. Thus, the fibrinogen-like domain when presented without additional regions of Tn-C was a good substratum for adhesion of CEWB cells even though it was unable to stimulate chondrogenesis. The largest recombinant Tn-C (Tn-260), although able to stimulate chondrogenesis, was less adhesive than control. These results demonstrate

that the ability of Tn-C to promote chondrogenesis is independent of its ability to support initial adhesion of CEWB cells.

Tn-C is mitogenic for some cell types [17]. In this study, only Tn-190 and Tn-ADC+ stimulated proliferation of CEWB cells. Ability to induce proliferation was not, however, essential for stimulation of chondrogenesis but it may help to achieve the highest possible levels. Mitogenic activity appears to reside within the fibrinogen-like domain, since this is the sole difference between Tn-190 (mitogenic) and Tn-FB− (non-mitogenic); it also appears to be suppressed by the presence of non-constitutive Fn type III-like repeats A, B and D.

Tn-C is present in early mesenchymal condensations of chicken long bone and stimulates chondrogenesis in culture. That the fibrinogen-like domain is necessary, but not sufficient, for stimulation of chondrogenesis was demonstrated. Other regions appear to be necessary possibly for providing the required spatial organization. Specific non-constitutive Fn type III-like repeats not usually expressed in chondrogenic regions also appear to cause partial suppression of Tn-C activity. These results emphasize the potential importance of isoform switching as a possible means of regulating Tn-C function. Stimulation of chondrogenesis by Tn-C is not caused simply by an effect on cell attachment while stimulation of CEWB cell proliferation by Tn-C is not necessary for, but enhances the chondrogenic capacity of Tn-C. These results demonstrate that multiple interactions between Tn-C and mesenchymal cells contribute to the role of Tn-C in the regulation of chondrogenesis.

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References

[1] Dessau, W., von der Mark, H., von der Mark, K. and Fischer, S. (1980) J. Embryol. Exp. Morphol. 57, 51–60.
[2] Kimata, K., Oioke, Y., Tani, K. and Shinomura, T. (1986) J. Biol. Chem. 261, 13517–13525.
[3] Muratoglu, S., Bachrati, C., Malpeli, M., Szabó, P., Neri, M., Dozin, B., Deák, F., Cancedda, R. and Kiss, I. (1995) Eur. J. Cell Biol. 68, 411–418.
[4] Chen, Q., Johnson, D.M., Haudenschild, D.R. and Goetinck, P.F. (1995) Dev. Biol. 172, 293–306.
[5] Mackie, E.J., Thesleff, I. and Chiquet-Ehrismann, R. (1987) J. Cell Biol. 105, 2569–2579.
[6] Mackie, E.J. and Murphy, L.I. (1998) Microsc. Res. Tech. 43, 102–110.
[7] Chuong, C.-M., Widelitz, R.B., Jiang, T.-X., Abbott, U.K., Lee, Y.-S. and Chen, H.-M. (1993) Prog. Clin. Biol. Res. 383, 465–474.
[8] Nies, D.E., Hemesath, T.J., Kim, J-H., Gulcher, J.R. and Stefansson, K. (1991) J. Biol. Chem. 266, 2818–2823.
[9] Jones, F.S., Hoffman, S., Cunningham, B.A. and Edelman, G.M. (1989) Proc. Natl. Acad. Sci. USA 86, 1905–1909.
[10] Fischer, D., Brown-Lüdi, M., Schulthess, T. and Chiquet-Ehrismann, R. (1997) J. Cell Sci. 110, 1513–1522.
[11] Fischer, D., Chiquet-Ehrismann, R., Bernasconi, C. and Chiquet, M. (1995) J. Biol. Chem. 270, 3378–3384.
[12] Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 61–68.
[13] Hamburger, V. and Hamilton, H.L. (1951) J. Morphol. 88, 49–92.
[14] Ahrens, P.B., Solursh, M. and Reiter, R.S. (1977) Dev. Biol. 60, 69–82.
[15] LaFleur, D.W., Chiang, J., Fagin, J.A., Schwartz, S.M., Shah, P.K., Wallner, K., Forrester, J.S. and Sharifi, B.G. (1997) J. Biol. Chem. 272, 32798–32803.
[16] Aukhil, I., Joshi, P., Yan, Y. and Erickson, H.P. (1993) J. Biol. Chem. 268, 2542–2553.
[17] End, P., Panaytou, G., Entwistle, A., Waterfield, M.D. and Chiquet, M. (1992) Eur. J. Biochem. 209, 1041–1051.