Adhesive Defect in Extracellular Matrix Tenascin-X-Null Fibroblasts: A Possible Mechanism of Tumor Invasion

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Extracellular matrix tenascin-X (TNX)-null mice, generated by disruption of the *Tnx* gene, display augmented invasion and metastasis of B16-BL6 melanoma tumor cells due to increased activities of matrix metalloproteinase (MMP)-2 and MMP-9. In this study, we investigated cell–matrix and cell–cell adhesions using TNX-null fibroblasts and wild-type fibroblasts. TNX-null fibroblasts exhibited a decreased attachment to fibronectin compared with that of wild-type fibroblasts. B16 melanoma cells were cocultured with wild-type or TNX-null fibroblasts, and the adhesion of B16 melanoma to the fibroblasts was assessed. B16 melanoma cells on wild-type fibroblasts proliferated and spread out in a horizontal direction, whereas those on TNX-null fibroblasts overlapped each other rather than migrating horizontally. These overlapping B16 melanoma cells on TNX-null fibroblasts peeled off faster than those on wild-type fibroblasts. To determine whether the decreased cell–matrix and cell–cell adhesions on TNX-null fibroblasts were due to increased MMP activity, the activities of MMPs in wild-type and TNX-null fibroblasts were compared by gelatinolytic assays. The analysis of MMPs from conditioned media demonstrated that almost the same levels of MMP activities were detected between wild-type and TNX-null fibroblasts. However, contrary to our expectations the activities of MMPs from conditioned media of B16 melanoma cells cocultured on TNX-null fibroblasts were rather reduced than those of B16 melanoma cells cocultured on wild-type. We concluded that the absence of TNX in the extracellular environment might play an important role in enhancement of the detachment of B16 melanoma cells.

Key words tenascin-X; matrix metalloproteinase; melanoma; extracellular matrix

Tenascin-X (TNX) is a large extracellular matrix glycoprotein belonging to the tenascin family. TNX was originally reported as a partial sequence encoded by gene X, found on the opposite strand of the steroid 21-hydroxylase gene[1,2] and located in the class III region of the major histocompatibility complex locus.[3—5] As mentioned above, TNX was initially discovered by genomic analysis but was also discovered independently by Lethias's group who had been focusing primarily on collagen fibril associated proteins and was given the name flexilin.[6,7]

TNX has characteristic domain structures, such as an N-terminal domain, heptad repeats, epidermal growth factor-like (EGF) repeats, fibronectin type III-like (FNIII) repeats, and a C-terminal globular fibrinogen domain. The five members of the tenascin family that have been identified in vertebrates, tenascin/cytotactin (tenascin-C, TNC), restrictin/J1-160/180 (tenascin-R, TNR), tenascin-X (TNX), tenascin-Y (TNY) and tenascin-W (TNW),[8—11] are made up of the same types of structural domains. TNX is ubiquitously expressed, especially in skeletal muscle and the heart. In skin and tissues of the digestive tract, the distribution of TNX is often reciprocal to that of TNC.[12] Interestingly, TNX is also highly expressed in the peripheral nervous system.[13,14]

Three functions of TNX have been proposed. TNX was found to be required for impeding the invasion and metastasis of tumor cells in experiments using TNX-deficient (-/-) mice.[15,16] TNX has also been shown to be involved in the regulation of collagen fibrillogenesis. Mao *et al.*[17] reported that TNX deficiency mimics Ehlers–Danlos syndrome, a heritable connective-tissue disorder, in mice through alteration of collagen deposition. Actually, five patients with Ehlers–Danlos syndrome were found to be deficient in TNX protein.[18,19] It has also been shown that TNX enhances the ability of VEGF family proteins to stimulate cell proliferation by association of VEGF family proteins with TNX.[20,21]

We have been particularly interested in the role of TNX in tumor invasion and metastasis. In a previous study, we found that the expression of TNX is down-regulated in high-grade astrocytomas as the grade of malignancy progresses in human.[22] Furthermore, TNX-/- mice showed a significant promotion of tumor invasion and metastasis due to increased activities of matrix metalloproteinase (MMP)-2 and MMP-9 compared with that in wild-type mice.[15] These findings suggest that a lack of TNX is sufficient for tumor invasion and metastasis and that reduced expression of TNX is a cause of tumor progression. Interestingly, in TNX and TNC double-knockout mice, TNX deficiency-induced tumor cell proliferation in the primary tumor site was suppressed by TNC deficiency.[16]

In the present study, we investigated the biological function of TNX in cell adhesion. We examined the ability of TNX-null dermal fibroblasts to attach to fibronectin and a highly invasive and metastatic cell line, B16 melanoma cells, compared with the ability of wild-type fibroblasts. We also carried out an experiment to determine whether the decreased adhesion in TNX-null fibroblasts seen both in cell–matrix and cell–cell interactions is due to enhanced MMP activity.

MATERIALS AND METHODS

Cells The generation of TNX-/- mice by homologous recombination using embryonic stem (ES) cells has been described previously.[15] The genetic background of wild-type and TNX-/- mice is a mixture of C57BL/6, CBA and ICR strains. All animal experiments were carried out in accor-

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dance with NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Hokkaido University. To prepare wild-type and TNX-null dermal fibroblasts, wild-type and TNX-/- mice were dissected on postnatal day 1 with scissors in phosphate-buffered saline (PBS)/2.5% trypsin (Becton Dickinson, Tokyo) supplemented with penicillin (200 units/ml) and streptomycin (200 μg/ml). A clump of cells was stirred for 30 min at 37 °C for dispersal of the cells. The cells were then centrifuged, collected, and cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). Mouse B16/lacZ melanoma cells that stably express the β-galactosidase were purchased from RIKEN Cell Bank (Tsukuba). The B16/lacZ melanoma cells were grown in DMEM/10% FBS according to the provided protocol. B16-BL6 melanoma cells were kindly provided by Dr. J. Hamada (Hokkaido University) and were maintained as monolayer cultures in DMEM/10% FBS.

Cell Attachment Assay One hundred microliters of fibronectin (Invitrogen, Tokyo) at the concentration of 1, 5, 10 or 25 μg/ml in PBS was added to each well of 96-well plates, incubated overnight at room temperature, and removed. Then PBS/0.1% BSA was added to each well and incubated at room temperature for 1 h, and the wells were then washed with PBS three times. Next, DMEM/0.1% BSA was added to each well and incubated at 37 °C for 1 h. Fifty microliters of wild-type or TNX-null fibroblast (5×10^3 cells/well) in DMEM/0.1% BSA was added to the fibronectin-coated wells and incubated for 30 min. Non-adherent cells were removed, and each well was washed with DMEM. Then 100 μl of the same medium was added, and this was followed by the addition of 10 μl of a cell counting kit (Wako Pure Chemical Industries, Osaka). After incubation at 37 °C for 2 h, the absorbance (450 nm) of formazan generated in the wells was measured using a Shimadzu CS-9300PC spectrophotometer (Shimadzu, Kyoto).

Coculture Assay Wild-type or TNX-/- fibroblasts were cultured into six-well culture dishes until 100% confluence in DMEM/10% FBS. After washing the fibroblasts with PBS, B16/lacZ melanoma cells were seeded on the fibroblasts at a density of fifty B16/lacZ melanoma cells/well in 3 ml of DMEM/3% FBS culture medium and cultured for 18 d. At 3-d intervals during the culturing period, the B16/lacZ melanoma cells cocultured on the fibroblasts were fixed in a solution containing 1% glutaraldehyde in PBS for 1 min at room temperature. After washing with PBS three times, the cells were stained at 37 °C for 30 min in a solution containing 1mM X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 1 mM $MgCl_2$ and 0.1% Triton X-100 in PBS. After washing with PBS, stained B16/lacZ melanoma cells were observed under a microscope (SZX12, Olympus, Sapporo).

Gelatin Zymography For *in vitro* analysis of MMP secretion, cells were cultured overnight in serum-free DMEM. For coculture of B16-BL6 cells on the fibroblasts, fibroblasts of each genotype were cultured until 100% confluence in DMEM/10% FBS, and then B16-BL6 melanoma cells were seeded on the fibroblasts at a density of 2.5×10^6 cells/10 cm culture dish. Two days later, the conditioned medium was changed to serum-free DMEM and further cultured overnight. The conditioned media from individual cell cultures were analyzed by gelatin zymography. The equal amounts of each conditioned media, based on cell numbers determined at the time of harvest were mixed with 2×gel loading buffer [0.1 M Tris–HCl (pH 6.8), 4% sodium dodecylsulfate (SDS), 20% glycerol and 0.1% bromophenol blue]. Samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) in gel containing 0.1% gelatin. The gels were washed for 1.5 h at room temperature in a solution containing 2.5% (v/v) Triton X-100 and subsequently incubated with a reaction buffer [50 mM Tris–HCl (pH 7.5), 5 mM $CaCl_2$, 0.02 mg/ml NaN_3, 1% Triton X-100 and 1 μM $Zn(CH_3COO)_2$] at 37 °C for 24 h. The gels were stained for 1 h with staining solution (0.1% Coomassie brilliant blue R250, 30% methanol and 10% acetic acid) and then destained in the same solution without Coomassie brilliant blue and dried directly between dialysis membranes.

RESULTS

In this study, the cell–matrix and cell–cell adhesive properties of fibroblasts derived from TNX-/- mice were examined by using an extracellular matrix substrate, fibronectin, and by coculturing with B16/lacZ melanoma cells, respectively.

To examine the cell–matrix adhesive properties of TNX-null fibroblasts on tissue culture plastic dishes that had been surface-coated with fibronectin, cell attachment assays were performed in the absence of serum. TNX-null fibroblasts exhibited a significant decrease in their ability to attach to fibronectin compared with that of wild-type fibroblasts (Fig. 1), indicating that TNX-null fibroblasts have a defect in their ability to adhere to a fibronectin substrate.

We have previously reported that promotion of the invasion and metastasis of a highly invasive and metastatic cell line, B16-BL6 melanoma cells, was observed in TNX-/- mice due to increased activity of MMP, compared with that in wild-type mice.[15] Thus, we speculated that the adhesion of melanoma to host cells in TNX-/- mice is weaker than that to wild-type host cells. In the present study, the adhesion of B16 melanoma to wild-type or TNX-null fibroblasts was assessed (Fig. 2). To enable easy chromogenic detection of the adhesion and migration of B16 melanoma cells on the wild-type or TNX-null fibroblasts, B16 melanoma cells expressing *Escherichia coli* β-galactosidase (B16/lacZ melanoma cells)

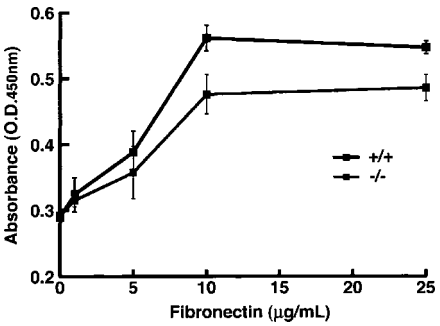

Fig. 1. Attachment of Wild-Type or TNX-Null Fibroblasts to Fibronectin-Coated Plates

Ninety-six-well plates were coated with various amounts of fibronectin. The fibroblasts of each genotype were added, and the number of attached cells was counted 30 min later using a cell counting kit. Data are expressed as the means of quadruplicate results with standard errors (mean±S.E.).

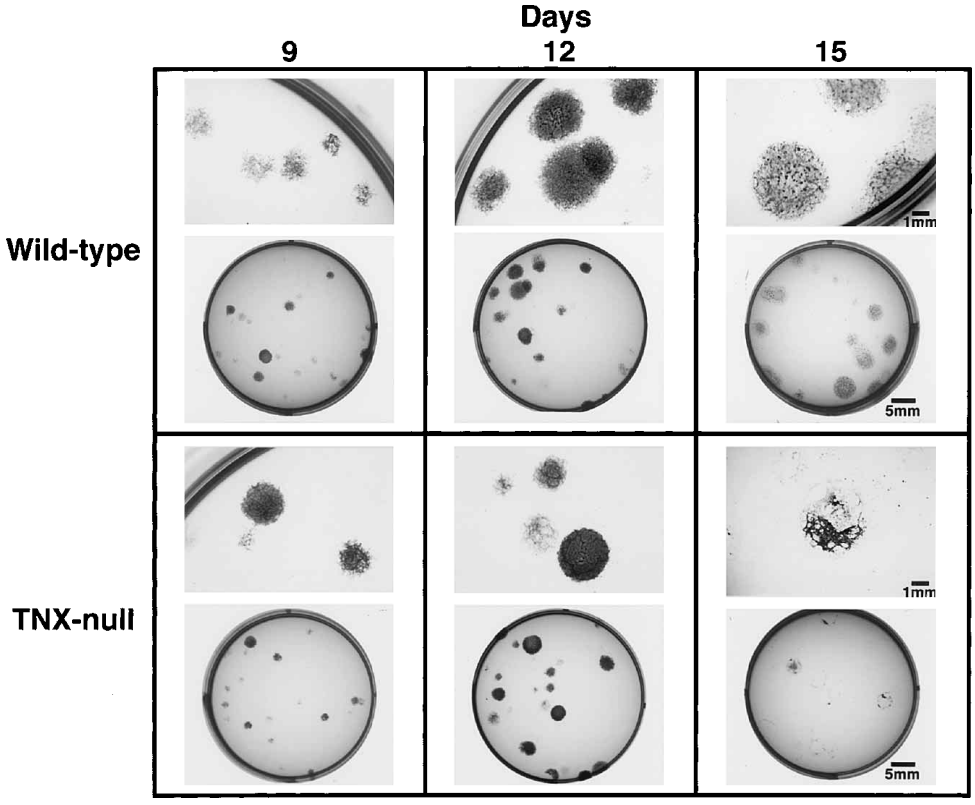

Fig. 2. Results of Coculture Assays of B16 Melanoma Cells on Fibroblasts of Each Genotype

B16/lacZ melanoma cells were seeded on wild-type or TNX-null fibroblasts. At 3-d intervals, the B16/lacZ melanoma cells cocultured on the fibroblasts were fixed and stained. Photographs taken at 9, 12 and 15 d after seeding of the melanoma cells on wild-type or TNX-null fibroblasts are shown here.

were used. As shown in Fig. 2, at 9 d after seeding on either wild-type or TNX-null fibroblasts, most of the B16/lacZ cells had adhered to the fibroblasts. However, the B16/lacZ cells proliferated and spread out in the horizontal direction on the wild-type fibroblasts, whereas those on the TNX-null fibroblasts overlapped each other rather than migrating horizontally. At 12 d after seeding, the B16/lacZ cells on the TNX-null fibroblasts had started to peel off. On the other hand, the B16/lacZ cells still adhered tightly to and were extended on the wild-type fibroblasts. At 15 d after seeding, the majority of B16/lacZ cells on the TNX-null fibroblasts had peeled off, whereas B16/lacZ cells on the wild-type cells had finally just begun to peel off. These results indicated that B16 melanoma cells on TNX-null fibroblasts tend to overlap each other rather than to spread on the fibroblasts and peel off with ease.

As stated in the above sections, decreased adhesion of TNX-null fibroblasts to fibronectin and B16 melanoma cells was observed. To determine whether the decreased cell–matrix and cell–cell adhesions on TNX-null fibroblasts were due to increased MMP activity in the TNX-null fibroblasts, the activities of MMPs secreted from wild-type and TNX-null fibroblasts were compared by gelatinolytic assays. As shown in Fig. 3, the results of gelatin zymography of conditioned media of wild-type and TNX-null fibroblasts showed that almost the same levels of MMP activities were detected. The relative band intensities of proMMP-2 from wild-type and TNX-null fibroblasts calculated by using of NIH image computer program were 1 and 0.735 ± 0.171 (means$\pm$S.E.), respectively. Further, the result for two groups were compared by a Student's *t*-test. No significant difference was detected

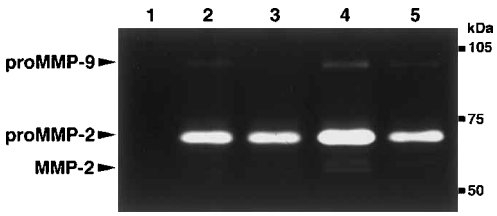

Fig. 3. MMP Activities by Wild-Type and TNX-Null Fibroblasts

Results of gelatin zymography of conditioned media of B16-BL6 melanoma cells (lane 1), wild-type fibroblasts (lane 2), TNX-null fibroblasts (lane 3), B16-BL6 melanoma cells cocultured on wild-type fibroblasts (lane 4), and B16-BL6 melanoma cells cocultured on TNX-null fibroblasts (lane 5). Note that B16-BL6 melanoma cells did not secrete MMPs. Gelatinolytic bands of proMMP-9, proMMP-2, and active MMP-2 are indicated by using arrow heads. A representative zymography from three separate experiments is shown.

between these two band intensities ($n=4$, $p>0.123$).

Subsequently, we carried out an experiment to determine whether the interaction of B16-BL6 (a highly invasive and metastatic B16 cell line) with the fibroblasts leads to the induction of MMP secretion from the fibroblasts and whether the extents of secretion from fibroblasts of the two genotypes are different. For this purpose, B16-BL6 cells were cocultured on wild-type or TNX-null fibroblasts, and then the conditioned medium was assessed by gelatin zymography. Since B16-BL6 melanoma cells do not secrete MMPs as shown by the results of a previous study[23] and as shown by the results of gelatin zymography in this study (Fig. 3, lane 1), the detected gelatinolytic activities in the conditioned medium would be derived from the fibroblasts. Consequently, as shown in Fig. 3, lanes 4 and 5 large amounts of proMMP-2

and small amounts of proMMP-9 and active MMP-2 were detected in both cultures. However, the relative band intensities of proMMP-2 from the conditioned media of B16-BL6 melanoma cells cocultured on wild-type fibroblasts and on TNX-null fibroblasts were 1 and 0.417 ± 0.088, respectively. Significant difference was detected between these two band intensities ($n=3$, $p<0.05$). Contrary to our expectations the activity of proMMP-2 was decreased in the conditioned medium of B16-BL6 melanoma cells cocultured on TNX-null fibroblasts compared with that on wild-type fibroblasts. The same tendency applied in the activities of proMMP-9 and active MMP-2. The activities of proMMP-9 and active MMP-2 were also reduced in the conditioned medium of B16-BL6 melanoma cells cocultured on TNX-null fibroblasts compared with those on wild-type fibroblasts. The relative band intensities of proMMP-9 from the conditioned media of B16-BL6 melanoma cells cocultured on wild-type fibroblasts and on TNX-null fibroblasts were 1.0 and 0.507 ± 0.169, respectively. Likewise the ralative band intensities of active MMP-2 were 1.0 and 0.240 ± 0.05, respectively. These data suggest that the easy peeling-off of B16 melanoma cells on TNX-null fibroblasts is due to the change of extracellular environment other than MMP activities through the absence of TNX (see Discussion).

DISCUSSION

We found in this study that TNX-null fibroblasts exhibit weaker adhesive properties to fibronectin and B16 melanoma cells than do wild-type fibroblasts. It is thought that this finding can explain why tumor invasion and metastasis are promoted in TNX-/- mice. However, we previously reported that the promotion of tumor invasion and metastasis is due to an increase in the production of MMPs in the skin.[15] This previously reported *in vivo* observation is not consistent with the present observations; that is, in the *in vitro* culture system the MMP activities in conditioned media from B16 melanoma cells cocultured on TNX-null fibroblasts were rather reduced compared with those from B16 melanoma cells cocultured on wild-type fibroblasts. At present, the relationship between the decreased MMP activities and weaker adhesive properties in TNX-null fibroblasts is not clear.

However, there are two possible reasons other than the involvement of MMPs for the easy peeling-off of B16-melanoma cells cocultured on TNX-null fibroblasts compared with that of wild-type fibroblasts. One possibility is that TNX acts directly as an adhesive or structural protein for the interaction of fibroblasts and B16 melanoma cells. Elefteriou *et al.*[24] demonstrated that MG63 and ECV304 cells adhere to purified TNX but do not spread and do not assemble actin stress fibers, indicating that TNX has adhesive activity, though it is weak. Thus, if TNX is absent in the extracellular space, adhesion between B16 melanoma and fibroblasts would be weaker. Another possibility is that TNX acts indirectly as a modulator of another extracellular matrix synthesis and/or organization. Actually, Mao *et al.*[17] reported that the amounts of type I collagen synthesized by TNX-/- and wild-type fibroblasts are similar but that TNX-/- fibroblasts can not deposit type I collagen into the cell-associated matrix. Another reported example in which the lack of an extracellular matrix influences the organization of another extracellular matrix is type VI collagen deficiency affecting the organization of fibronectin in the extracellular matrix of cultured fibroblasts.[25] Therefore, if TNX is lost, the synthesis and/or organization of another extracellular matrix protein would be altered, thus affecting the adhesive properties of the fibroblasts.

In conclusion, our study has demonstrated that TNX plays a role in the regulation of cell–cell and cell–matrix interactions. Further studies are needed to elucidate the biochemical basis for the decreased adhesion in TNX-null fibroblasts and to characterize the receptors and changes in intracellular signaling that are involved in the phenotypic differences in TNX-null fibroblasts.

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