Tenascin-X Induces Cell Detachment through p38 Mitogen-Activated Protein Kinase Activation

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Extracellular matrix glycoprotein tenascin-X (TNX) is the largest member of the tenascin family. In this study, we investigated the adhesive properties of TNX and the signaling pathway to be induced to mouse fibroblast L cells on TNX substrate. Approximately 45% of evaluable cells used in the cell adhesion assay were attached to purified TNX but did not spread and were rounded on TNX. The remaining 55% of cells were detached from the TNX substrate and were floating in the conditioned medium. In rounded cells on TNX, phosphorylation of focal adhesion kinase (FAK) was diminished compared with that in cells on control phosphate buffered saline (PBS). To better understand the pathways that lead to the detachment of cells on the TNX substrate, we examined phosphorylation of p38 mitogen-activated protein (MAP) kinase. Phosphorylation of p38 MAP kinase was observed in the rounded cells on TNX in a dose-dependent manner, and the maximum effect was observed at 30 min on TNX. Inhibition of p38 MAP kinase α expression by RNA interference partially suppressed the TNX-induced cell detachment. These results suggest that the p38 MAP kinase is a major mediator of TNX-induced cell detachment.

Key words tenascin-X; p38 mitogen-activated protein kinase; cell detachment

The extracellular matrix (ECM) has an important regulatory function in tissue homeostasis and is critically involved in cell proliferation, migration, differentiation, and survival.[1] As adhesive substrates, the ECM components such as fibronectin, collagen and laminin facilitate attachment, spreading and formation of focal adhesions through the cell surface receptors including integrins.[2] On the other hand, some components of the ECM can promote cell rounding and detachment. So far, tenascin-C (TNC),[3] thrombospondin-1[4] and SPARC (secreted protein acidic and rich in cysteine)[5] have been identified as members of the class of ECM proteins with anti-adhesive properties.

TNC belongs to the tenascin family of ECM modular glycoproteins. Four members of the tenascin family have been identified in vertebrates: tenascin-C (TNC), tenascin-R (TNR), tenascin-X (TNX) (known as tenascin-Y in birds), and tenascin-W (TNW).[6] Tenascin family members consists of the same types of structural domains, including a cysteine-rich segment at the amino terminus, epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a fibrinogen (FG)-like domain at the carboxyl terminus. Huang *et al.*[7] demonstrated a novel mechanism by which TNC impairs the adhesive function of fibronectin through its binding to the 13th fibronectin type III repeat of fibronectin and showed that this binding results in the inhibition of association of fibronectin with a receptor syndecan-4. Other members of the tenascin family have also been reported to have some of the basic anti-adhesive properties of TNC.[8—10]

TNX is the largest member of the tenascin family. Complete deficiency of TNX in humans leads to a rare recessive form of Ehlers-Danlos syndrome (EDS). The skin of TNX-deficient patients is markedly lax with poor recoil properties and shows easy bruising.[11—13] The clinical findings of TNX deficiency are similar to those of the classic type of EDS. However, TNX-deficient patients lack atrophic scars as well as delayed wound healing, which are major diagnostic criteria for the classic type of EDS.[12] TNX haploinsufficiency is associated with joint hypermobility type of EDS.[14]

Previous studies have indicated several functional properties of TNX, including collagen fibrillogenesis,[15,16] collagen deposition,[17] and development and maintenance of elastic fibers.[18]

As for the adhesive properties of bovine TNX in which the 10th FNIII repeat possesses an Arg-Gly-Asp (RGD) putative cell attachment sequence, Elefteriou *et al.*[9,19] demonstrated that osteosarcoma (MG63) and bladder carcinoma (ECV304) cells adhere to bovine TNX weakly and that do not spread and do not assemble actin stress fibers. They also identified two weakly adhesive sites in bovine TNX: the FG domain and the RGD sequence within the FNIII repeat mediated by integrin receptors, $\alpha v\beta 3$ and $\beta 1$ integrin subunit, respectively.

Upon adhesion, adhered anchorage-dependent cells transmit survival signals from the matrix into the cells. Loss of adhesion and anchorage leads to anoikis, the subset of apoptosis triggered by inadequate or inappropriate cell–matrix contacts.[20] Resistance to anoikis is a critical requirement for invasion and metastasis in cancers derived from epithelial cells. As for the signaling events involved in anoikis, p38 mitogen-activated protein (MAP) kinase (or stress-activated MAP kinase (SAPK)) has been suggested to play a role in anoikis.[21,22] Four p38 paralogues, p38α (or MAPK14), p38β, p38γ (or SAPK3), and p38δ (or SAPK4), have been identified in mammals. They are known to have distinct functions depending on the cellular type and differentiation state contexts.[23]

In this study, we investigated the adhesive properties of purified mouse TNX, which does not contain an RGD putative cell attachment sequence, unlike bovine TNX. We found that about half of the mouse fibroblast L cells did not spread and were rounded on TNX and that more than half of the mouse L cells were detached from the mouse TNX substrate

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and were floating in the conditioned medium. We were interested to clarify whether the p38 MAP kinase signaling transduction pathway participates in the loss of adhesion induced by TNX. Our results showed that at least p38α isoform is involved in TNX-induced detachment of cells.

MATERIALS AND METHODS

Cell Cultures Mouse fibroblast L cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% bovine calf serum (JRH Biosciences, U.S.A.), 0.15% $NaHCO_3$, penicillin (10 units/ml) and streptomycin (10 mg/ml).

Cell Adhesion Assay Adhesion assays were performed in six-well plates coated with 10 μg/ml fibronectin (Millipore/Chemicon, Temecular, California, U.S.A.), or with 10 μg/ml affinity-purified TNX from wild-type mouse liver as described previously.[24,25] As negative controls, the wells were coated with the same amount of extract [referred to as Ctrl(TNX−/−)] purified from TNX-deficient (TNX−/−) mice[26] or with phosphate-buffered saline (PBS) [referred to as Ctrl(PBS)]. Ctrl(TNX−/−) was extracted from livers of TNX−/− mice by the same procedures as TNX from wild-type mice had been purified. Mouse fibroblast L cells, at 80% confluence, were trypsinized, suspended in serum-free DMEM (3.3×10^6 cells/ml), and placed in suspension culture for 1 h at 37 °C. Three hundred microliters of cell suspension was added to each well in six-well plates, and the cells were allowed to attach for 30 min. Then adherent cells were trypsinized, suspended in PBS, and counted directly under a light microscope in four different fields in at least three independent experiments. Non-adherent cells were collected and counted similarly.

Immunostaining Cover glasses were coated with 10 μg/ml fibronectin or TNX and then placed in six-well plates. According to the procedures described in "Cell Adhesion Assay" section, 300 μl of L cells suspended in serum-free DMEM (1×10^6 cells/well) was added to each well coated with ECM, and the cells were allowed to attach for 1 h. The cover glasses were then taken out from the wells. After removal of non-adherent cells by washing with PBS, attached cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100. The cover glasses were stained with rhodamine-phalloidin (Invitrogen, Carlsbad, CA, U.S.A.) and anti-vinculin antibody (Sigma, Saint Louis, MO, U.S.A.). For detection of vinculin immunoreactivity, anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (Millipore/Chemicon) was used. Nuclear DNA was visualized with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg/ml) (Invitrogen). The cells were then observed using a fluorescent microscope, All-In-One (BZ-8000) (Keyence, Osaka, Japan).

Western Blot Analysis As described in the "Cell Adhesion Assay" section, six-well plates were coated with each ECM, and L cells suspended in serum-free DMEM (2×10^6 cells/well) were added to each well. Then the cells were allowed to attach to each ECM for 30 min. The cells were washed with PBS and then harvested and homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (NP-40), 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Complete™ protease inhibitor set (Roche Diagnostics, Indianapolis, IN, U.S.A.). The homogenate was centrifuged and the supernatant was collected and stored at −80 °C. The protein concentration was determined by a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, U.S.A.). Equal amounts of sample proteins were mixed with Laemmli buffer, boiled for 5 min, and subjected to a 10% SDS-PAGE under reducing conditions for Western blot analysis. Western blot analysis was performed as previously described.[27] After electrophoretic transfer of proteins to ECL nitrocellulose filters (GE Healthcare, Buckinghamshire, England), the filters were blotted with antibodies against the indicated protein kinases or phosphorylated forms of these kinases. These antibodies used were as follows: rabbit polyclonal phospho-specific focal adhesion kinase (FAK) antibodies (Phospho Y397) (Abcam, Cambridge, England); mouse monoclonal FAK antibody (clone 4.47) (Millipore/Upstate, Lake Placid, NY, U.S.A.); rabbit polyclonal phospho-specific p38 MAP kinase antibodies (Thr180/Tyr182) (Cell Signaling, Danvers, MA, U.S.A.), rabbit polyclonal p38 MAP kinase antibodies, which detect total levels of endogenous p38 MAP kinase protein (Cell Signaling); and mouse monoclonal p38α MAP kinase-specific antibody (L53F8) (Cell Signaling). For an internal loading control, mouse monoclonal anti-β-tubulin antibody (Sigma) or anti-actin antibody (Millipore/Chemicon) was used. Then the filters were washed three times with TBST containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20 and incubated with anti-mouse or anti-rabbit Alexa Flour 680-conjugated (Invitrogen, Tokyo, Japan) or IRDye 800-conjugated IgG (Rockland Immunochemicals, Gilbertsville, U.S.A.) followed by visualization using an infrared imaging system (Odyssey, LI-COR, Lincoln, U.S.A.).

siRNA Experiment RNAi experiments for mouse p38 MAP kinase α were carried out with small interfering RNAs (siRNA). The target sequence for mouse p38 MAP kinase α purchased from Nippon EGT (Toyama, Japan) was 5′-GACUGUGAGCUCAAGAUUCdTdT-3′ (sense) and 5′-GAAUCUUGAGCUCACAGUCdTdT-3′ (antisense). The control siRNA sequence was 5′-CGUACGCGGAAUACUUCGAdTdT-3′ (sense) and 5′-UCGAAGUAUUCCGCGUACGdTdT-3′ (antisense), which bears no homology with relevant mouse genes. Synthetic siRNA was transfected into L cells plated in 6-well plates (1×10^5 cells/well) by using Lipofectamine (Invitrogen, Tokyo, Japan) or Lipofectoamine™RNAiMAX (Invitrogen) at a final concentration of 100 nM following the manufacturer's instructions. After 24 h, the medium was changed to DMEM with serum and the transfected cells were cultured for 48 h. Then cells were trypsinized, suspended in serum-free DMEM (3.3×10^6 cells/ml), and placed in suspension culture for 1 h at 37 °C. Fifty microliters of cell suspension was collected and centrifuged, and then cells were homogenized in lysis buffer as described in the "Western Blot Analysis" section. Three hundred microliters of cell suspension was used for cell adhesion assay with using 24-well plates coated with each ECM as described in the "Cell Adhesion Assay" section.

RESULTS

TNX-Induced Cell Detachment To examine the adhesive properties of mouse TNX, which does not contain an RGD putative cell attachment sequence, mouse TNX was affinity-purified from an adult liver as described previously.[24,25] Then we investigated adhesive properties of the purified TNX to mouse fibroblast L cells (Fig. 1A). Cells were serum-starved, trypsinized, and plated on TNX, fibronectin (FN), and PBS [Ctrl(PBS)]. In addition, as a negative control, the cells were plated on the same amount of extract [Ctrl(TNX−/−)] purified from TNX-deficient (TNX−/−) mice by the same procedures as TNX from wild-type mice had been purified. The rate of L-cell attachment over a period of 30 min was determined. More than 80% of evaluable cells on FN and Ctrl(PBS) as well as Ctrl(TNX−/−) were attached. However, only 45% of cells tested were attached on TNX. The remaining 55% of cells were detached from TNX. Subsequently, the morphology of the attached cells was observed as shown in Fig. 1B. On the FN substrate, the cells were well spread and contained numerous stress fibers, while the cells on TNX were round and stress fibers were absent. This is readily visible in the phalloidin-stained cells in Fig. 1Bb compared to Fig. 1Ba. Furthermore, FN allowed the formation of prominent mature focal adhesions that can be readily visualized using an antibody against vinculin, whereas L cells on the TNX substrate failed to form focal adhesions (Fig. 1Bd compared to Fig. 1Bc). In the focal adhesion, FAK provides a dock for focal adhesion proteins and signaling molecules to transduce extracellular signals initiated by the binding of integrin to the ECM. It is known that the tyrosine residue at position 397 (Y397) of FAK is auto-phosphorylated upon integrin engagement.[28] Next, we investigated whether Y397 is phosphorylated in association with cell adhesion on TNX by Western blot analysis using the phospho-Y397-specific FAK antibody (Fig. 1C). The immunoblots demonstrated strong phosphorylation of Y397 on FN. In contrast, phosphorylation of Y397 was weakly detected on TNX (40% phosphorylation of that on FN). These results indicated that mouse TNX suppresses the spreading of newly plated L cells and promotes partial rounding and detachment of the cells.

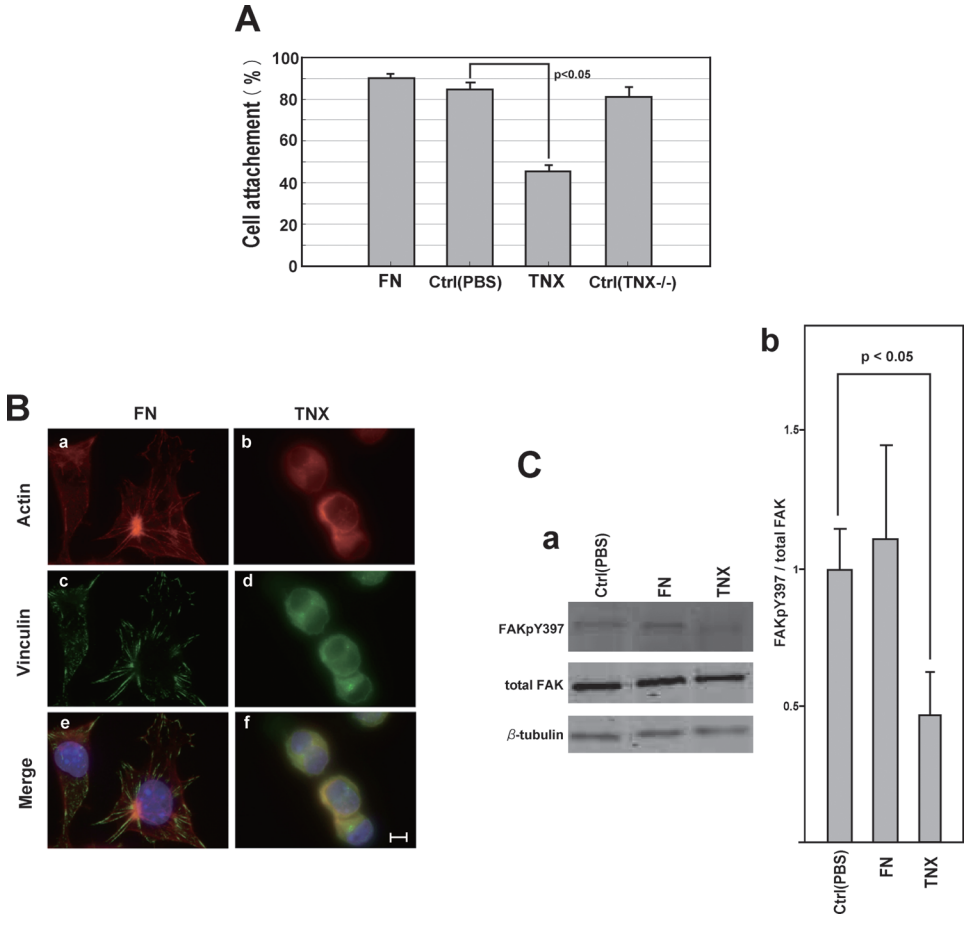

Fig. 1. Compromised Cell Adhesion on TNX Substrate

(A) Cell attachment assay. Six-well plates were coated with ECM molecules (FN, fibronectin; TNX, tenascin-X) at 10 μg/ml. As a negative control, plates were coated with the same amount of extract purified from TNX-deficient (TNX−/−) mice [Ctrl(TNX−/−)] or phosphate-buffered saline [Ctrl(PBS)]. L cells were plated on coated wells and incubated at 37 °C for 30 min. After incubation, the plates were washed and the number of adherent cells was counted. The number of attached cells on each substrate is expressed with respect to input cell number (100%). Means±standard deviation of triplicated experiments were calculated, and statistical analysis was performed using the Mann–Whitney *U* test. (B) Phalloidin and vinculin staining of L cells on TNX substrate. L cells plated on FN (a and c) and on TNX (b and d) were labeled with rhodamine-phalloidin (a and b, Actin) and an antibody against vinculin (c and d, Vinculin). Nuclear DNA was visualized with DAPI (e and f). Furthermore, pictures (a) and (c) and pictures (b) and (d) were merged with (e) and (f), respectively. Bar, 10 μm. (C) FAK phosphorylation on tyrosine residue 397 plated on TNX substrate. (a) Whole cell lysate attached on FN, TNX, and Ctrl(PBS) was analyzed for FAK expression (total FAK) and specific phosphorylation on tyrosine residue 397 (FAKpY397) by Western blotting. As an internal loading control, mouse monoclonal anti-β-tubulin antibody was used (β-tubulin). (b) Densitometric analysis of three experiments for phospho-Y397 FAK (FAKpY397) over FAK expression (total FAK). The values are shown relative to the levels in the control cells on Ctrl(PBS) (1.0). Means±standard deviation of triplicated experiments were calculated, and statistical analysis was performed using the Mann–Whitney *U* test.

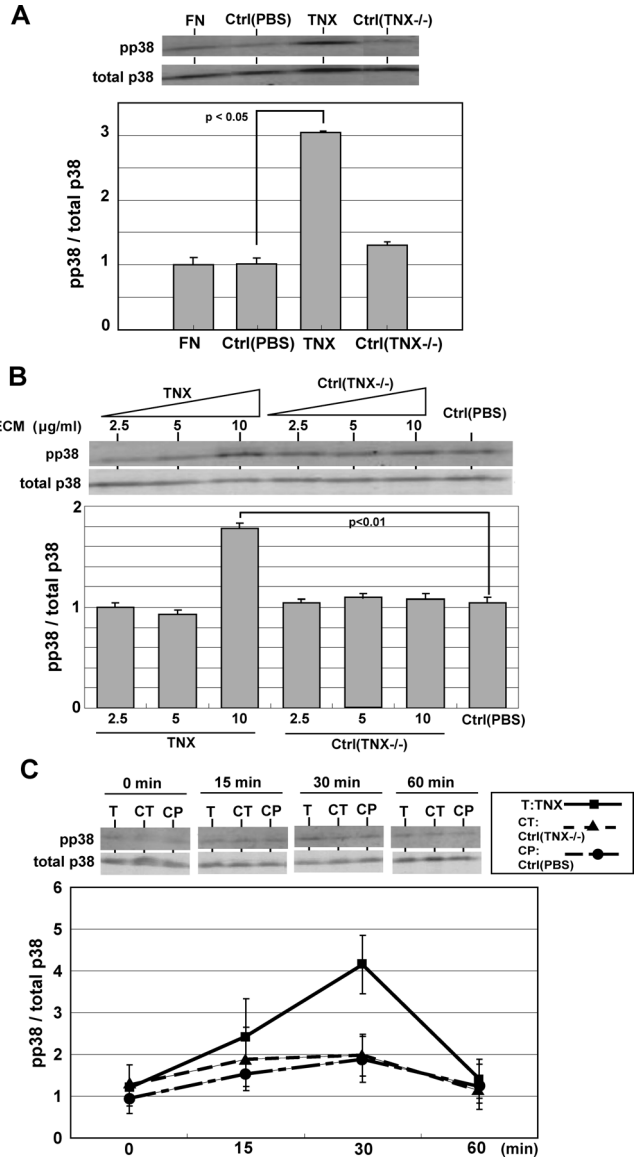

Fig. 2. p38 MAP Kinase Activation on TNX Substrate

(A) TNX induces p38 MAP kinase activity. (Upper panel) L cells were plated on the indicated ECM (FN, fibronectin; TNX, tenascin-X). As a negative control, phosphate-buffered saline [Ctrl(PBS)] or the same amount of extract purified from TNX-deficient (TNX−/−) mice [Ctrl(TNX−/−)] was used. After 30 min, the cells were assayed for p38 MAP kinase phosphorylation (pp38) and p38 MAP kinase expression (total p38) by Western blot analysis. (Lower panel) Densitometric analysis of three experiments for phosphorylated p38 MAP kinase over p38 MAP kinase expression. The values are shown relative to the levels in the cells on FN (1.0). Means±standard deviation of triplicated experiments were calculated, and statistical analysis was performed using the Mann–Whitney *U* test. (B) Activation of p38 MAP kinase by TNX in a concentration-dependent manner. (Upper panel) Wells were coated with the indicated concentrations of TNX, Ctrl(TNX−/−) or Ctrl(PBS), and L cells were plated. After 30 min, the cells were assayed for p38 MAP kinase phosphorylation (pp38) and p38 MAP kinase expression (total p38) by Western blot analysis. (Lower panel) Densitometric analysis of three experiments for phosphorylated p38 MAP kinase over p38 MAP kinase expression. The values are shown relative to the levels in the cells on 2.5 μg/ml TNX (1.0). Means±standard deviation of triplicated experiments were calculated, and statistical analysis was performed using the Mann–Whitney *U* test. (C) Activation of p38 MAP kinase by TNX at different time points. (Upper panel) Wells were coated with TNX (T), Ctrl(TNX−/−) (CT) or Ctrl(PBS) (CP), and L cells were plated at the indicated time points (0 to 60 min). The levels of activated p38 MAP kinase (pp38) and total p38 MAP kinase expression (total p38) were determined by Western blot analysis. (Lower panel) Densitometric analysis of three experiments for phosphorylated p38 MAP kinase over p38 MAP kinase expression. The values are shown relative to the levels in the control cells on Ctrl(PBS) at 0 min (1.0). Means±standard deviation of triplicated experiments were calculated.

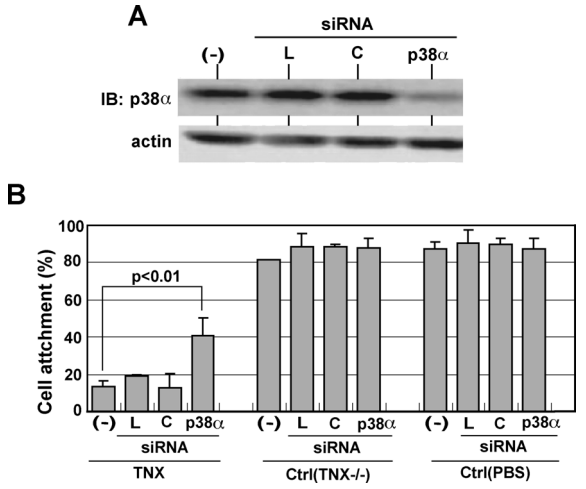

Fig. 3. Suppression of TNX-Induced Cell Detachment by Knocking Down p38α MAP Kinase Expression

(A) Knockdown of the expression of p38α MAP kinase in L cells by siRNA. Mouse p38 MAP kinase α-specific siRNA (p38α) or control siRNA (C) was transfected into L cells by using Lipofectamine. As a negative control, transfection was performed by using only Lipofectamine without any siRNA (L). (−) indicates that transfection was not performed in L cells. After 72 h of culture following transfection, cell lysates were prepared and analyzed for p38α MAP kinase expression (p38α) by Western blotting. As an internal loading control, mouse monoclonal anti-actin antibody was used (actin). (B) Cell attachment assay on TNX substrate using the p38α knockdown cells. p38α knockdown cells (p38α) as well as negative control cells (−, L, and C) were plated on 24-wells coated with TNX for 30 min. As negative controls, cells were coated with [Ctrl(TNX−/−)] or [Ctrl(PBS)]. Then the number of adherent cells was counted as shown in Fig. 1A.

Activation of p38 MAP Kinase Phosphorylation in L Cells on TNX Substrate It is known that p38 MAP kinase activity is required for cell detachment.[21] We reasoned that if p38 MAP kinase plays a role in the TNX-induced cell detachment, cells attached to TNX should display higher levels of p38 MAP kinase activity than that of cells attached to FN. We examined p38 MAP kinase activities in cells that were plated and allowed to attach for 30 min on TNX, FN, Ctrl(PBS), and Ctrl(TNX−/−). As shown in Fig. 2A, we found that TNX induces a strong increase in phosphorylation of p38 MAP kinase compared to that induced by FN, Ctrl(PBS), or Ctrl(TNX−/−). Furthermore, unlike Ctrl(TNX−/−), the L cells attached to TNX showed a concentration-dependent activation of p38 MAP kinase phosphorylation (Fig. 2B). Interestingly, we also found a time-dependent increase in p38 MAP kinase activity in L cells attached to TNX, with maximal activation occurring around 30 min (Fig. 2C). In contrast, activation of p38 MAP kinase was not found in L cells attached to Ctrl(TNX−/−) or Ctrl(PBS) at any time point. These findings clearly indicate that TNX contributes to the activation of p38 MAP kinase in L cells.

Involvement of p38α MAP Kinase in TNX-Induced Cell Detachment Next, we examined whether p38 MAP kinase is involved in cell detachment induced by the TNX substrate. For this purpose, we performed knockdown of endogenous p38α MAP kinase by siRNA prior to the cell adhesion assay on the TNX substrate, since p38α MAP kinase is most ubiquitously expressed among the four mammalian p38 isoforms[29] and is thought to be involved in the regulation of many fundamental cellular processes, including differentiation, proliferation, anoikis and apoptosis.[30] After knocking down the expression of p38α MAP kinase in L cells (Fig.

3A), the cells were allowed to attach for 30 min on TNX, Ctrl(TNX−/−) or Ctrl(PBS), and the attached cells were counted. As shown in Fig. 3B, knockdown of p38α MAP kinase suppressed TNX-induced cell detachment, and cell attachment was partially restored. However, in the case of knockdown using control siRNA, which failed to decrease p38α MAP kinase expression, suppression of TNX-induced cell detachment was not observed. These results indicated that p38 MAP kinase, at least the α isoform, is involved in TNX-induced cell detachment.

DISCUSSION

Rosen *et al.*[21] have demonstrated that rat intestinal epithelial cell detachment caused by culture in suspension triggers p38 MAP kinase-dependent overexpression of Fas ligands, leading to anoikis. On the other hand, we showed in this study that L cell attachment to the TNX substrate leads to activation of p38 MAP kinase followed by detachment of the cells from TNX. The signaling pathways from attachment to TNX to activation of p38 MAP kinase phosphorylation and also from activation of p38 MAP kinase to cell detachment have not been elucidated. However, the fact that type I collagen mediates matrix metalloproteinase-13 (MMP-13) expression *via* αvβ1 and α2β1 integrins and the fact that collagen-dependent induction of MMP-13 expression requires p38 MAP kinase activity[31] might suggest that TNX-induced p38 MAP kinase activation causes altered expression of some MMP(s), resulting in cell detachment from the TNX substrate.

We showed that p38 MAP kinase, at least the α isoform, is involved in TNX-induced cell detachment (see Fig. 3). We also attempted to perform knockdown of other isoforms of endogenous p38 MAP kinase (p38β, p38γ, p38δ) by siRNAs. Unfortunately, we were not able to inhibit the expression of p38β and p38δ MAP kinases by the siRNAs. However, since we could slightly decrease the expression of p38γ MAP kinase, a cell attachment assay was done using these p38γ MAP kinase-knockdown cells on the TNX substrate. Results of our preliminary experiment showed that knockdown of p38γ MAP kinase fails to suppress TNX-induced cell detachment (data not shown). Therefore, TNX-induced cell detachment might be caused by a certain p38 isoform-specific activation.

Elefteriou *et al.*[9] have reported that MG63 and ECV304 cells cannot spread and cannot assemble actin stress fibers on bovine TNX, which possesses an RGD sequence in the FNIII repeat. As shown in the present study, mouse TNX, which does not contain the RGD sequence, has the same anti-adhesive properties as those of bovine TNX. Therefore, TNX has the characteristic that it cannot spread cells and then leads to cell detachment regardless of the presence or absence of the RGD sequence. Further studies are needed to assess which region within the TNX molecule is responsible for cell detachment and to determine the cell surface receptors that interact with TNX to transmit the intracellular signals to p38 MAP kinase. More detailed analysis of the signal transduction pathway downstream of p38 MAP kinase leading to cell detachment should also be carried out.

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