Molecular Mechanism of Tenascin-C Action on Matrix Metalloproteinase-1 Invasive Potential

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The aim of the current study was to confirm that tenascin-C large splice variant (TNC320) stimulates matrix metalloproteinase-1 (MMP-1) expression and to elucidate molecular mechanisms underlying this activation. The analysis of gene expression in cultured cells grown under different conditions indicated significant increases of MMP-1 mRNA steady-state levels in the cells treated with TNC320 (200%) compared with TNC220 (100%) and bovine serum albumin (BSA), which served as controls. Gel electrophoresis results demonstrated augmented MMP-1 protein in cells cultured with TNC320, whereas slight up-regulation was noticed in cells treated with TNC220 or fibronectin. Reverse transcriptase polymerase chain reaction results demonstrated significantly higher levels of MMP-1 gene expression in TNC320 cultured cells than in all other treatment groups. The result was confirmed by examining the level of MMP-1 promoter transactivation by different extracellular proteins. Data demonstrated 30-fold activation of MMP-1 promoter by TNC320 treatment in comparison with other treatments (TNC220 or fibronectin) and BSA as a control. Both invasion and collagenase activity assays demonstrated a 3-fold difference in the cells treated with TNC320 in comparison with the control. MMP-1 was quantified by enzyme-linked immunosorbent assay as well. Experiments with constitutively active expression kinases indicated that MMP-1 expression induced by TNC320 was associated with mitogen-activated protein kinase (MAPK) cascade activation. Culture with TNC320 resulted in more than 2-fold activation of MMP1-luciferase in the presence of mitogen-activated protein kinase kinase kinase 1 and also 2-fold down-regulation in the presence of mitogen-activated protein kinase kinase 1. We hypothesize that tenascin-C stimulates invasion via up-regulation of MMP-1 expression through activation of MAPK cascade signaling. Exp Biol Med 232:515–522, 2007

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Introduction

Chondrosarcoma is the second most common malignancy in bone and results from unregulated growth of mesenchymal stem cells with varying degrees of cartilaginous differentiation (1, 2). The tumor is locally invasive and subsequently metastasizes via hematogenous spread to the lungs. We have shown a marked prognostic significance for recurrence in patients with high levels of matrix metalloproteinase-1 (MMP-1) gene expression in patients with chondrosarcoma (3). Other studies have reported the involvement of MMPs in tumor growth and spread. It is believed that this enzyme facilitates cellular egress from tumor matrix (4, 5).

Tenascin-C (TNC) is an adhesion-modulating extracellular matrix glycoprotein that is highly expressed in tumor stroma and stimulates tumor cell proliferation (6). The increased expression of TNC and its proliferative and anti-adhesive properties have been correlated with metastatic potential in several malignancies (7–9). Two major TNC slice variants have been identified as 220-kDa (TNC220) and 320-kDa (TNC320) bands on Western blot analysis. It is possible that the individual TNC splice variants play distinctly different roles in cell signaling. Previous studies have reported an association between malignant cells and the relatively increased production of the large TNC splice variant (6, 8, 10). We have reported that the relative expression of the large TNC splice variant may correlate with malignancy and poor clinical outcome in human chondrosarcoma (11). This led to the hypothesis that chondrosarcoma matrix may function as a dynamic reciprocity in which the tumor cell secretes a specific matrix and in turn is modulated by the matrix to increase the metastatic potential of the cell (3, 4). While TNC has been shown to correlate with patient survival and metastasis, the mechanism of TNC-induced metastasis has to be yet determined.

Cytoplasmic signaling is induced upon cell adhesion to adhesive extracellular matrix molecules and includes activation of the mitogen-activated protein kinase (MAPK) pathway involving phosphorylation of threonine and tyrosine residues by mitogen-activated protein kinase kinase kinase (12). The latter is often deregulated in cancer cells (13, 14). Studies have shown links between TNC and altered MAPK signaling (15). It has been shown that collagen-dependent induction of MMP-13 in dermal fibroblasts requires p38 activity and is inhibited by activation of extracellular signal-regulated kinase 1 (ERK1)/ERK2. Therefore, the balance between the activity of ERK1/ERK2 and MAPK pathways appears to be crucial in regulation of MMP-13 expression in dermal fibroblasts (16). Another study demonstrated that up-regulation of MMP-1 expression in fibroblasts involves MAPK pathways, and these are integrated at the level of the promoter (17). This leads to the hypothesis that activation of the MAPK pathway by extracellular TNC signals is involved in MMP-1 transcription.

The aim of the current study was to determine if TNC220 and TNC320 differentially stimulate MMP-1 gene expression *in vivo* and thereby increase the invasive potential of cultured human chondrosarcoma cells. A secondary goal was to determine the involvement of MAPK in this activation process.

Materials and Methods

Plasmids. TNC320 expression vector (pNUT-HxB.L) and TNC220 and TNC320 proteins were gifts from Dr. Harold Erickson (18). Human MMP-1 promoter-driven luciferase reporter construct (nucleotides –525 to +15) and deletion constructs from the same promoter –300/+15; –91/+15; –41/+15 were gifts from Dr. Ralf Janknecht (19).

Cell Culture (Alginate Beads). The chondrosarcoma cell line was cultured in a monolayer until confluent and subsequently encapsulated and recultured in alginate beads. The beads contain exogenous glycoproteins, added as determined by the experimental design. The cells were cultured at a density of 1×10^6 cells/ml, as previously described (20). Purified human fibronectin (FN) was diluted in phosphate-buffered saline (PBS) to a concentration of 2 μg/ml (Chondrex, Seattle, WA). Human TNC320 and TNC220 were diluted to a concentration of 10 μg/ml. Previous investigators had determined the specific concentrations of each glycoprotein that most accurately mimic the *in vivo* extracellular matrix (ECM; Ref. 21).

Cell Recovery. Culture medium, alginate, and cells were harvested on culture days 0, 3, 7, and 14. Culture medium was collected and stored at –70°C. Alginate beads were dissociated by chelation with 28 m*M* EDTA/0.15 *M* NaCl recovery buffer (pH 7.0). Cells were then pelleted by centrifugation at 1000 *g* for 5 mins, and the EDTA/alginate supernatant containing noncell-associated ECM was collected and stored at –70°C. For Western blot analysis, the cell pellet was resuspended in 1 ml of PBS and subjected to ultrasonic lysis (Heat Systems, Farmingdale, NY).

Cell Culture. The chondrosarcoma cell line JJ012 was provided by Dr. Joel Block (11). The chondrosarcoma cells were cultured in monolayer until semiconfluent. Media consisted of Dulbecco's modified Eagle's medium (DMEM)/MEM supplemented with F12, 10% fetal bovine serum, 25 μg/ml ascorbic acid, 100 ng/ml insulin, 100 nM hydrocortisone, and 1% penicillin/streptomycin antibiotic mixture (Gibco, Grand Island, NY).

Western Blotting. SDS-polyacrylamide gel electrophoresis and Western blotting were performed, as described elsewhere (22, 23). MMP-1 protein was detected by mouse anti-MMP1 monoclonal antibody from Chemicon International (Temecula, CA). MMP-1 (Sigma-Aldrich, St. Louis, MO) from human fibroblasts was used as a positive control.

Transient Transfections and Reporter Gene Assay. Expression plasmids containing mitogen-activated protein kinase kinase 1 (MEK1) and mitogen-activated protein kinase kinase kinase 1 (MEKK1) promoter with a downstream luciferase reporter construct were obtained from Clontech (Mountain View, CA). Cells were seeded at a density of 2×10^5 cells/ml in 6-well plates 24 hrs prior to transfection and incubated at 37°C for 24 hrs. Transfection was performed using Lipofectamine 2000 reagent from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Six hours posttransfection the lipofectin complex was removed and substituted with fresh growth media. Cells were harvested for luciferase assay (Promega, Madison, WI) 24 hrs after transfection, and luciferase activity was quantified using a TD20/20 luminometer (Turner BioSystems, Sunnyvale, CA). All values were normalized to protein content or to renilla luciferase activity (24). MMP-1-luc activity was measured by this method as well.

Quantitative Polymerase Chain Reaction (Q-PCR) Analysis. RNA was extracted from cell pellets using the RNAeasy extraction kit following the manufacturer's instructions (Qiagen, Valencia, CA). Lightcycler probe design software (Roche, Basel, Switzerland) was used to design primer pairs for MMP-1 and the housekeeping gene β2-microglobulin. Real-time reverse transcriptase (RT)-PCR was performed on a Lightcycler (Roche) using the Lightcycler RNA Amplification Kit SYBR Green I, a one-step RT-PCR protocol (Roche). The MMP-1 transcript was quantitated using a method of relative quantification (25). A standard curve was generated from known concentrations of an RNA sequence homologous to MMP-1 to ensure identical amplification efficiency at all dilutions. Quantification was determined by comparing sample MMP-1 and β2-microglobulin values to the standard curve. Final results were generated from the ratio of the MMP-1 gene to β2-microglobulin.

Standard PCR Analysis. RNA was reverse transcribed and prepared as per manufacturer's instructions for standard PCR amplification using the Thermoscript RT PCR Kit (Invitrogen). Primers for either MMP-1 or the house-

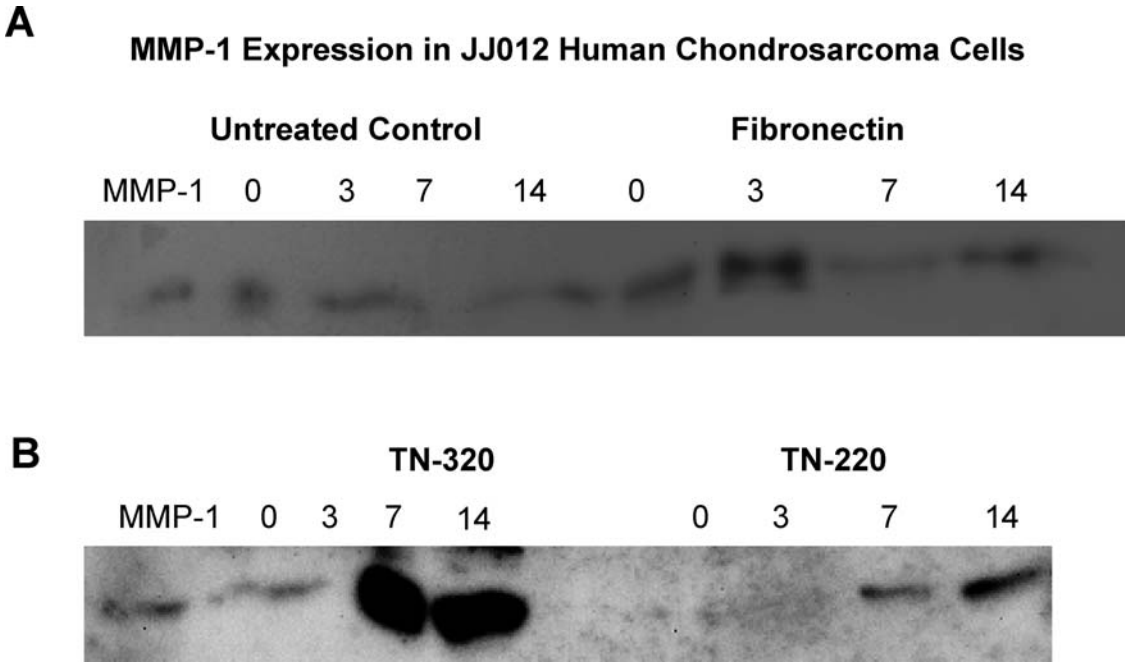

Figure 1. Western blot analysis for JJ012 fractions from beads representing conditioned media collected after 0, 3, 7, and 14 days. Upper panel (A) represents control (nontreated cells) and fibronectin treatments. Lower panel (B) represents TNC320 and TNC220 treatments with final concentration of TNC320/220 (10 μg/ml) and FN (2 μg/ml). The figure demonstrates augmented expression of MMP-1 protein on the 7th and 14th days in the cells cultured with TNC320 ($P < 0.01$), whereas slight up-regulation is noted in cells treated with TNC220 or FN. The left lane labeled MMP-1 represents a positional control.

keeping gene glyceraldehyde-3-phosphate dehydrogenase were designed as described above using primer design software. Reactions were as follows: denaturation at 94°C for 5 mins, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 mins. Finally, an extension at 72°C for 7 mins was performed. The PCR products were electrophoresed for 45 mins at 120V on a 2% agarose/Tris-acetate-EDTA gel and photographed (3). The PCR primers were the following: forward 5′-CATTGATGGCATCCAAGC-3′ and reverse 5′-CCGGACTTCATCTCTGT-3′.

Purification of Tenascin. TNC220 and TNC320 proteins were purified from a transfected CHO cell line and BHK cell line, respectively (18). The proteins from the conditioned media were precipitated by adding ammonium sulfate (22.1 g/100 ml). The precipitated proteins were pelleted by centrifugation and resuspended in 0.02 M ammonium bicarbonate (pH 8). The protein solution was centrifuged and separated by Superose 6 (Amersham Biosciences, Inc., Piscataway, NJ) and subsequently Mono-Q ion exchange chromatography. Protein yield and purity were assessed by protein assay, silver staining, and Western blot. The protein assays and Western blots were performed as described in the Western Blotting Analysis section below using a TNC primary antibody (1 μg/ml; Abcam, Cambridge, MA).

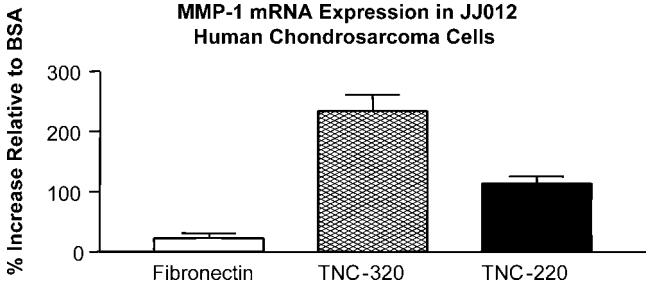

Figure 2. QPCR analysis demonstrates that TNC320 stimulation of JJ012 cultured cells (7 days) significantly increased MMP-1 mRNA expression compared with treatment with TNC220, FN, and BSA ($P = 0.0009$). TNC220 generated significantly more MMP-1 mRNA than BSA alone ($P = 0.007$).

Collagenase Activity Assay. Collagenase assay was performed using a collagenase assay kit from Chondrex, Inc. (Redmond, WA) following the manufacturer's instructions. Bovine type II collagen was used as a substrate (3). Invasion assay was performed using QCM96-Well Collagen-Based Cell Invasion Assay from Chemicon International according to the manufacturer's instructions (26). MMP-1 enzyme-linked immunosorbent assay (ELISA) was carried out using an MMP-1 ELISA kit from Calbiochem (San Diego, CA) (25).

Densitometry and Statistical Analysis. All experiments were performed in triplicate, and $P < 0.05$ was considered significant. Data analysis was performed using one-way analysis of variance (ANOVA) and unpaired *t*-test (GraphPad Prism; GraphPad Software, Inc., San Diego, CA). Densitometry was performed on the standard RT-PCR gels using NIH Imaging software (Scion Corp., Frederick, MD). Data analysis was performed using one-way ANOVA

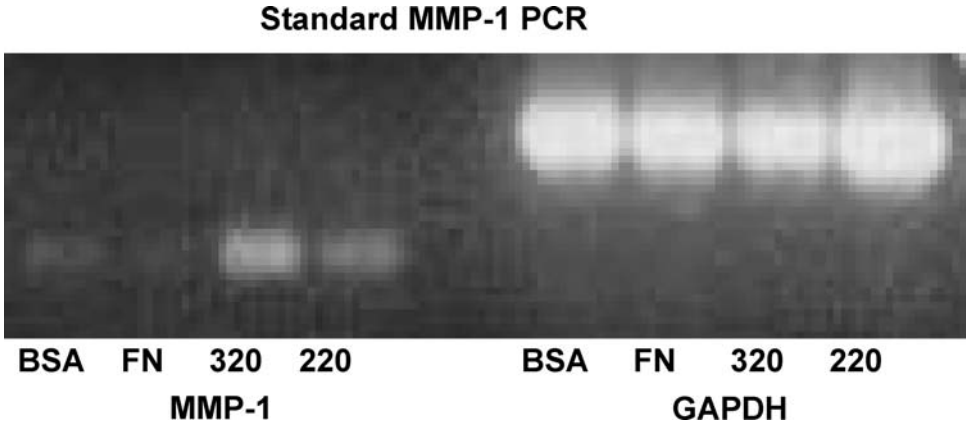

Figure 3. RT-PCR results show that TNC320-treated cells (7 days) expressed significantly higher levels of MMP-1 than all other treatment groups ($P < 0.0001$), including TNC220. The latter showed an increase compared with the BSA control ($P = 0.032$) but demonstrated significantly less of an effect than treatment with TNC-320. Treatment with FN resulted in no significant changes in comparison with control and other treatments ($P = 0.1766$).

and unpaired *t*-test (GraphPad Prism; GraphPad Software, Inc.). The resulting Western blot band densities were analyzed with Kodak ID Image Analysis software. The relative protein expression was determined by calculating the ratio of protein expression of the splice variants and FN-treated samples to those treated with bovine serum albumin (BSA).

Results

TNC effect on MMP-1 protein level was evaluated by examining the ability of TNC220, TNC320, and FN to stimulate the transcription and activity of MMP-1 in human chondrosarcoma cells (Fig. 1). The figure demonstrates augmented expression of MMP-1 (Fig. 1A) on the 7th and 14th days in the cells cultured with TNC320 ($P < 0.01$), whereas only slight up-regulation was noted in cells treated with TNC220 or FN (Fig. 1B). Analysis of gene expression in cultured cells grown under different conditions (7 days) indicated a significant increase of MMP-1 mRNA expression in TNC320-treated cells (200%) compared with TNC220 ($P = 0.0009$) (100%) or FN and BSA (Fig. 2). The difference between TNC220 and TNC320 mRNA expression was not as great as that observed for protein expression.

RT-PCR results depicted in Figure 3 demonstrate significantly higher levels of MMP-1 gene expression in TNC320-cultured cells (7 days) than in all other treatment groups, including TNC220 ($P < 0.0001$). While TNC220 treatment resulted in a statistically elevated level of MMP-1 expression in comparison with control BSA treatment (30%) ($P = 0.032$), it was still less compared with the cells treated with TNC320 (50%). Densitometry analysis of the bands from the Western blot showed consistent results in both PCR results (Fig. 1C). Treatment with FN resulted in no significant change in MMP-1 expression ($P = 0.1766$).

This result was confirmed by examining the effect of TNC splice variants on the level of transactivation of MMP-1 promoter by different extracellular proteins *in vivo*. The results demonstrated a 30-fold activation of MMP-1 promoter by TNC320 treatment in comparison with control and other treatments (Fig. 4) ($P < 0.0001$).

Invasion assays were performed with JJ012 human

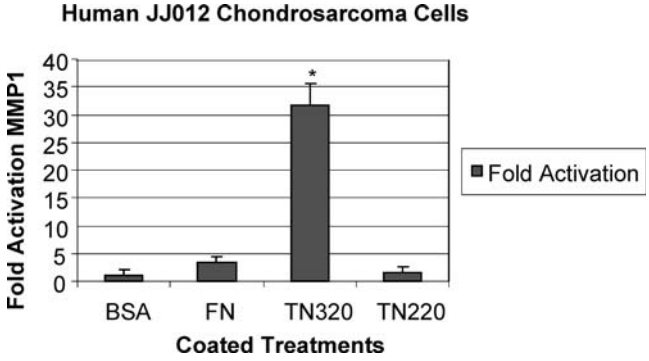

Figure 4. Transactivation of MMP-1 luciferase construct in transiently transfected JJ012 human chondrosarcoma cultured cells (2×10^5 cells/well). Cells were seeded on top of precoated treatments of TNC320/220 (10 μg/ml), FN (2 μg/ml), and BSA (1%). TNC320 treatment causing transactivation proved to be statistically significant ($P < 0.0001$).

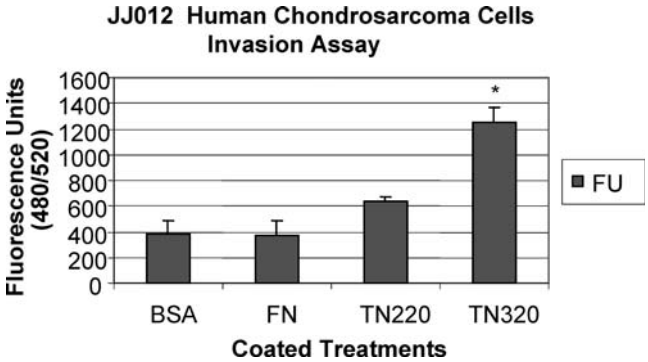

Figure 5. Invasion assay performed on JJ012 human chondrosarcoma cell line. An almost 3-fold difference is observed in the cells treated with TNC320 in comparison with control ($P < 0.0001$).

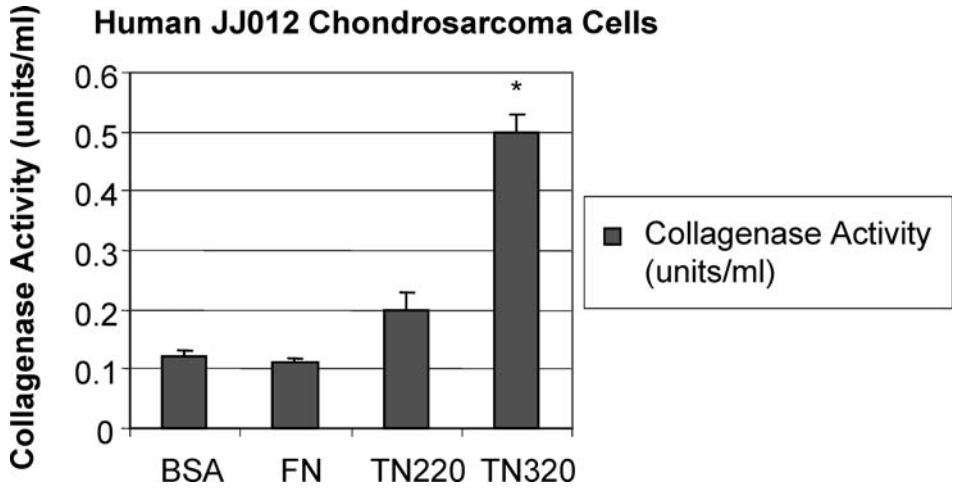

Figure 6. Collagenase activity in JJ012 human chondrosarcoma cells with TNC320/220 (10 μg/ml), FN (2 μg/ml), and BSA (1%). An almost 3-fold difference in collagenase activity was detected in the cells pretreated with TNC320 ($P < 0.01$).

chondrosarcoma cells with collagen precoated chambers to determine if up-regulation of MMP-1 resulted in increased *in vitro* invasion (Fig. 5). A 3-fold difference was observed in the cells treated with TNC320 in comparison with control ($P < 0.0001$). Collagenase activity was measured in JJ012 cells exposed to extracellular proteins TNC320/220 (10 μg/ml), FN (2 μg/ml), and BSA (1% w/v) (Fig. 6). An almost 3-fold difference in collagenase activity was detected in the cells pretreated with TNC320 ($P < 0.01$). Additionally, MMP-1 protein was quantitated by ELISA in the same cell fractions, and this is shown in Figure 7. In combination, these data indicate that MMP-1 gene expression, protein levels, and invasiveness were up-regulated in cells cultured in the presence of TNC320.

Experiments with constitutively active expression kinases indicate that no differences in MMP-1-luc activation were observed with TNC220, FN, or BSA (Fig. 8A) in the presence of kinase vectors. On the other hand (Fig. 8B), the culture with TNC320 resulted in more than 2-fold activation of MMP-1-luc activity in the presence of MEKK1 and almost 2-fold down-regulation of MMP-1-luc in the presence of MEK1 ($P < 0.0007$).

Discussion

Tumor cell invasion and metastasis are the result of a multistep mechanism involving intravasation, transport, and extravasation. One aspect of this cascade that is common to all solid tumors is the ability of cells to escape the constraints of the ECM. For cartilaginous tissues, the ECM molecules consist of collagen, proteoglycans, and glycoproteins, such as tenascin, FN, and laminin. Metalloproteinases have been identified with the ability to degrade collagen and other ECM proteins. We have previously reported that MMP-1 gene expression is an independent predictor of survival in chondrosarcoma, leading to the hypothesis that collagenase serves to facilitate

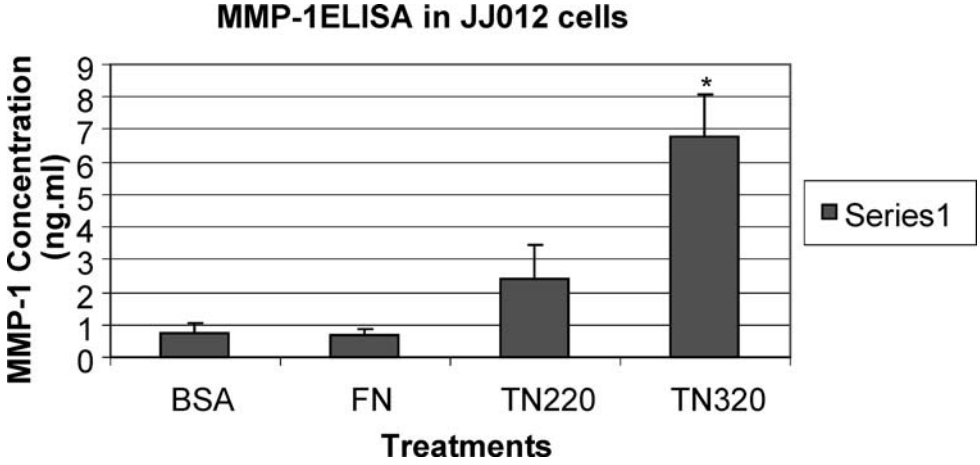

Figure 7. MMP-1 ELISA was performed on JJ012 human chondrosarcoma cultured cells in conditioned media (diluted with the assay buffer in the ratio 1:2.5 and collected on the 7th day). MMP-1 levels of TNC320 samples (6.68 ng/ml) were elevated in comparison with other treatments (FN and BSA) and that of TNC220 (2.4 ng/ml). The values are the average of triplicate measurements on the same preparations; the error bars are standard deviations ($P < 0.0004$).

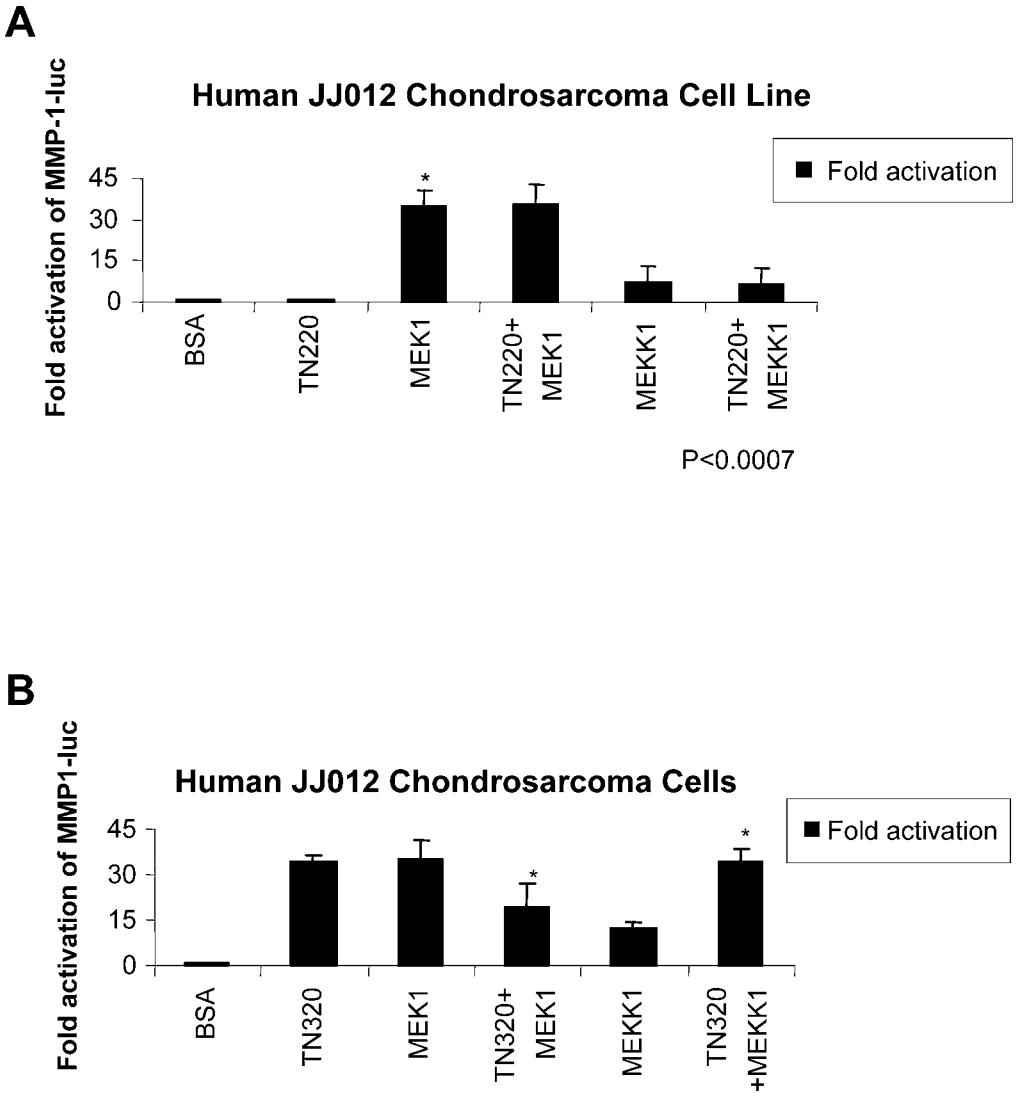

Figure 8. Transactivation of the MMP-1-luc construct in transiently transfected JJ012 human chondrosarcoma cultured cells (2×10^5 cells/well). Cells were seeded on top of precoated treatments of TNC320/220 (10 μg/ml), FN (2 μg/ml), and BSA (1%). Constitutively expressed kinases (500 ng/μl) were added to the transfection complex in equimolar concentrations (A). TNC220 treatment did not have any effect on up-regulation of MMP-1 luciferase, in the presence of either MEK1 or MEKK1. On the contrary, TNC320 treatment (B) caused up-regulation of MEKK1 (more than 2-fold), and almost 2-fold down-regulation of MEK1 ($P < 0.0007$).

cell egress from the tumor matrix. It has been reported that TNC stimulates MMP-1 expression in other experimental systems (3, 4). This has led us to postulate that in chondrosarcoma the matrix may function as a dynamic reciprocity in which tumor cells secrete a specific matrix and in turn are modulated by that matrix to alter biologic behavior of the cell. This hypothesis opens up possibilities of altering ECM composition or altering the interaction with cellular elements to manipulate the activity of MMPs and subsequently metastasis. Since TNC is known to be co-expressed with MMPs in other tissues, the aim of this study was to determine if TNC320 might play a role in stimulating MMP-1 expression and thereby increase the invasive potential of cultured human chondrosarcoma cells. As a secondary goal, we sought to elucidate molecular mechanisms of TNC action on MMP-1 and invasive potential. Our results in this study demonstrate that TNC320 stimulates MMP-1 gene expression and protein synthesis. Analysis of cells grown on corresponding matrix proteins (BSA, TNC220, TNC320, and FN) showed a 30-fold activation of MMP-1 gene expression in TNC320-treated cells versus other ECM proteins. We observed a 3-fold increase in collagenase activity with TNC320, which was associated with increased *in vitro* invasiveness. These data suggest that TNC320 is involved in activating the MMP cascade and possibly the initiation of extravasation. The results of this study demonstrate that TNC320 (but not TNC220) stimulates MMP-1 expression through an MAPK pathway. Taking into consideration our experimental results demonstrating that TNC320 up-regulates MMP-1 expression and that MAPK pathway modules are involved in this up-regulation, it may be that MAPK pathway activation is a

necessary prerequisite for TNC-induced altered MMP-1 expression. This is consistent with previous studies, which provided evidence that distinct extracellular signals leading to up-regulation of MMP-1 expression involve MAPK pathways and are integrated at the level of the promoter (17). Observations of MAPK up-regulation of MMPs in cancer progression have been recognized previously (27). Moreover, this type of regulation by the matrix has been reported in other systems (26). Interestingly, it is not only that extracellular TNC influences intracellular MAPK and MMP regulation but also that MMPs, MAPK, and integrins regulate TNC transcription (28) and deposition (29). Hence, this is an interesting example of dynamic reciprocity.

It has been observed that the biologic sequelae of TNC is dependent on the splicing pattern. The differentiation of functions between the TNC splice variants is largely unknown. The smaller TNC isoform may provide a separate and distinct cellular response, whereas the larger isoform, preferentially expressed in malignant tissue, likely plays a role in cell egress and metastasis (30, 31). Other malignancies have noted differences in TNC splice variant expression, and in breast and thyroid this has involved the preferential synthesis of the large splice variant by malignant tissues (32–34). Recently investigators have reported an accumulation of the large TNC splice variant in the matrix of prostate carcinoma (35). As each of these systems is further explored, a better understanding of the role of splicing in this protein will be attained.

We hypothesize that TNC stimulates invasion via up-regulation of MMP-1 expression and that this mechanism involves MAPK cascade signaling. When the molecular mechanisms and function of TNC action on MMP up-regulation are established, translation to preclinical trials that either modify or down-regulate TNC expression can be considered. It is also conceivable that lessons learned from these studies can be applied to different types of malignancies.

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