Role of fibrillar Tenascin-C in metastatic pancreatic cancer

JIAN CHEN¹, ZHIYU CHEN², MING CHEN, DAJIANG LI, ZHIHUA LI, YAN XIONG, JIAHONG DONG and XIAOWU LI

Hepatobiliary Surgery Institute, Southwest Hospital, Third Military Medical University, Chongqing, P.R. China

Received November 11, 2008; Accepted January 7, 2009

DOI: 10.3892/ijo_00000228

Abstract. Interaction of cancer cells with stroma cells facilitates tumor progression by rebuilding the existing extracellular matrix (ECM) microenvironment. In the tumor, upregulation of Tenascin-C (Tn-C) expression potentially can alter tumor behavior. However, the molecular mechanisms by which tumor-stroma interactions affect the tumor microenvironment have not been well characterized. In this study, we analyzed the expression of fibrillar Tn-C (fTn-C) in human metastatic pancreatic cancers. After co-culturing two pancreatic cancer cell lines, highly metastatic BxPc3 cells and non-metastatic PaCa2 cells, with stromal fibroblasts (SF), we evaluated the roles of matrix metalloproteinase 2 (MMP-2) activation and SF in promoting Tn-C organization. Next, we evaluated whether fibrillar Tn-C promotes pancreatic cancer cell movement using cell adhesion and migration assays. Finally, we observed the relationship between MMP-2 activation and fTn-C formation in vivo by injecting the BxPc3 and PaCa2 cells into nude mice. We found that fTn-C was increased in metastatic pancreatic cancer. The fTn-C expression correlated with MMP-2 activity. In the in vitro co-culture, fTn-C organization was found only in BxPc3/SF co-cultures, and required the participation of active MMP-2. The fTn-C reduced cell adhesion and promote pancreatic cancer cell migration by decreasing the adhesive interactions between integrin α6β1 and the ECM. The in vivo tumorigenesis analysis showed that the fTn-C formation and active MMP-2 were significantly increased in the BxPc3 tumors, compared to the PaCa2 tumors. These results demonstrate that Tn-C deposition into the ECM requires participation of active MMP-2 and SF. The deposited Tn-C could promote pancreatic cancer progression.

Introduction

Tenascin-C (Tn-C) and fibronectin (FN) are glycoproteins of the extracellular matrix (ECM) that interact with each other and with other matrix molecules including collagen and heparan sulfate proteoglycans. FN is processed into an insoluble fibrillar matrix to which Tn-C binds either directly or indirectly via proteoglycans that bridge the two proteins (1). Both proteins contain sites that are recognized by cell surface receptors whose occupation allows the ECM to regulate the adhesion, differentiation, growth, and migration of cells. Tn-C interferes with the cell adhesive function of FN either by binding to FN and restricting access to its integrin binding sites (2), or by binding to cell receptors and altering their responsiveness to FN (3,4). Tn-C is transiently expressed during fetal development, and is absent or greatly reduced in most adult tissues. It is increased in some pathological conditions, including inflammation, wound healing and cancer. Tn-C is highly expressed in the majority of malignant solid tumors, including those originating from the brain, breast, colon, prostate and pancreas. Tn-C is secreted by both cancer cells and stromal cells (5). The most prominent effects of Tn-C are anti-adhesion (6) and inhibition of cell attachment (7), both of which favor cancer cell motility and invasion. However, it is unknown whether Tn-C incorporation into the ECM is required for either effect.

Activation of the stromal microenvironment of a tumor is an important step in cancer growth and progression. However, the mechanisms by which cancer cells activate stromal cells are not well understood. In a previous study, we found that interaction between cancer cells and stromal fibroblasts is required for pancreatic cancer metastasis (8). Interaction of cancer cells with stromal cells can result in secretion of cancer-specific ECM proteins, cytokines, growth factors and matrix remodeling enzymes (8-10). Evidence is accumulating that some cancer-specific ECM proteins may influence cancer progression. Both tumor and stromal cells secrete matrix degrading enzymes, including matrix metalloproteinases (MMPs), that can cleave Tn-C. MMP-7 and MMP-2 cleave the large variant of Tn-C in the alternatively spliced fibronectin type III repeat domain (11).

Although an intimate relationship clearly exists between the growing tumor and surrounding stromal environment, the molecular mechanisms by which tumor-stroma interactions modify the tumor microenvironment have not been well characterized. To evaluate whether this interaction is required for the incorporation of Tn-C into the tumor microenvironment,
we analyzed the expression of Tn-C in human metastatic pancreatic cancers. In addition, we evaluated the effect of co-culturing two pancreatic cancer cell lines, highly metastatic BxPc3 cells and non-metastatic PaCa2 cells, with stromal fibroblasts on Tn-C organization and MMP-2 activity. Finally, we used an in vivo model of tumorigenesis in nude mice to evaluate the roles of MMP-2 activation and SF in promoting Tn-C organization.

Materials and methods

Tissue samples. Twenty pancreatic adenocarcinoma patients from the Hepatobiliary Surgery Institute (Southwest Hospital, Third Military Medical University, Chongqing, P.R. China) were selected for this study with randomized and case-pair controls (including age, tumor size, location, and classification). The tumor specimens included 6 metastatic pancreatic cancer specimens (liver metastasis) and 10 non-metastatic pancreatic cancer specimens obtained from surgery, as well as 4 liver metastasis specimens obtained from formalin-fixed, paraffin-embedded tissues (Table I). Each pancreatic tumor specimen was reviewed by pathologists. The protocol was approved by the Institutional Review Board and the patients gave written consent.

Antibodies and reagents. Antibodies used were anti-MMP-2 (Oncogene Science), anti-Tn-C (Sigma, St. Louis, MO) and anti-FN (Sigma). All chemicals used were of analytic grade (Oncogene Science), anti Tn-C (Sigma, St. Louis, MO) and anti-MMP-2 (Sigma). Antibodies used were anti-MMP-2 and anti-FN (Sigma). All chemicals used were of analytic grade and were purchased from Chongqing Biotech Co. (China).

Immunohistochemistry. The details of the procedure have been described previously (3). Briefly, the deparaffinized sections were trypsinized [0.05% trypsin in 0.05% Triton X-100 in Tris-buffered saline (Triton-TBS)] for 20 min and blocked with 10% goat serum in Superblock, a proprietary blocking reagent containing a mammalian derived protein (Pierce). Each section was incubated separately with monoclonal antibodies (anti-Tn-C at 20 μg/ml and anti-MMP-2 at 10 μg/ml) at 4˚C for 18-24 h. After washing four to five times (15 min each) with Triton-TBS, the slides were processed in the automated stainer (Ventana) according to the manufacturer’s instructions. The immunoperoxidase-3, 3-diaminobenzidine-stained slides were subsequently counterstained with hematoxylin and mounted on a coverslip. Normal pancreatic tissue samples were used as controls.

Cell cultures. The metastatic human pancreatic carcinoma cell line BxPc3 and non-metastatic human pancreatic carcinoma cell line PaCa2 were purchased from the American Type Culture Collection (Manassas, VA). SF was isolated from pancreatic carcinoma tissues from surgery in our Institute. Epithelial cell contamination was excluded by light microscopy. These cells were maintained in DMEM with 10% fetal calf serum (FCS).

Immunofluorescence microscopy. Glass coverslips were cleaned with 5 M HCl and treated with 2% solution of dimethylchlorosilane in trichloroethane. The cells were seeded on the coverslips for the indicated times, then fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, permeabilized with 0.2% Triton X-100 for 1 min, rinsed several times with PBS, and incubated with the primary antibodies for 1 h. Fluorescein isothiocyanate (FITC) or rhodamine (Rho)-conjugated secondary antibodies were applied for another 45 min. The coverslips were mounted onto slides and observed by epifluorescence with a Zeiss microscope. For co-culture experiments, the coverslips were put on the bottom of 24-well plates; the SF cells were grown onto glass coverslips for 2 h, and then cancer cells were added for another 48 h of culture. The staining was performed as described above.

Gelatin zymography. MMP-2 and MMP-9 activity was analyzed by 10% sodium dodecyl sulfate (SDS)-gelatin substrate gel. The serum-free conditioned medium was collected and subjected to gelatin-SDS-PAGE electrophoresis. The gels were treated with 2.5% Triton X-100 at 37˚C for 30 min to remove SDS and then incubated at 37˚C for 16 h in substrate buffer (50 mM Tris-HCl and 5 mM CaCl2 at pH 8.0). The gels were stained with 0.15% Coomassie blue R-250 (Bio-Rad) in 50% methanol, 10% glacial acetic acid at room temperature for 20 min, and then destained in the same solution without Coomassie blue. Enzyme activity was identified as clear gelatin-degrading bands against the blue background.

Isolation of Tenascin-C. Pancreatic cancer cells co-cultured with SF were grown for 48 h, after which the cells were washed three times with hypotonic buffer (0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 8.0) for 20 min each time on ice, followed by one short wash with PBS. The plates with the remaining the extracellular matrix proteins were then collected by adding 5 ml of PBS. The protein was purified by sequential chromatography over a gelatin-agarose column (Sigma) to remove fibronectin, and then a Sepharose 4B column (10-ml bed volume; Pharmacia) coupled with a monoclonal antibody against human Tn-C. The column was eluted with 50 mM diethylamine in H2O, pH 11. The fractions were stored at -20˚C for further experiments.

Cell lysis and Western blotting. The cells were lysed in NP40 lysis buffer (1.5% Nonidet P40, 150 mM NaCl, 0.2% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 1 mM Na2VO4, 50 mM NaF). Protein concentrations were determined with a BCA protein assay kit (Pierce). For Western blotting, cell lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to nylon membranes. Membranes were probed with primary antibodies followed by peroxidase-labeled secondary antibodies and visualized by enhanced chemiluminescence detection system (Amersham) according to the manufacturer’s instructions.

Adhesion assay. Cell adhesion assays were performed in 96-well reduced glutathione (GSH)-coated plates (Pierce) that were pretreated with SuperBlock. The GSH plates were first coated with ECM proteins [10 μg/ml FN, 10 μg/ml collagen I (Col I), 30 μg/ml laminin-1 (Lm-1), and 200 ng/ml Tn-C] overnight at 4˚C. Cells were detached with 0.025%
trypsin and 0.53 mM EDTA (Life Technologies). Cells were then resuspended in serum-free DMEM at a cell density of 5.0x10^5 cells/ml. For each well, 100 μl of cell suspension was seeded and allowed to attach for 2 h at 37˚C. Non-adherent cells were removed; adherent cells were fixed with glutaraldehyde [3% (v/v) in PBS] and stained with crystal violet [3% (w/v) in methanol] and absorbance was measured at 595 nm.

Migration assay. Cell migration assays were carried out as previously described. Briefly, Transwell filters (Costar, Cambridge, MA) were coated on the lower side with purified ECM proteins (10 μg/ml FN, 10 μg/ml Col I, 200 ng/ml Tn-C, and 30 μg/ml Ln-1 in the presence or absence of GoH3). Cells (5x10^4 in 500 μl of serum-free DMEM containing 0.1% BSA) were added to the upper chamber and allowed to migrate for 4 h at 37˚C. Cells that migrated to the bottom surface of the filter were fixed with methanol, stained with crystal violet, and counted. Each substrate was repeated in triplicate wells, and, within each well, counting was done in four randomly selected microscopic fields at magnification x300.

Orthotopic model for pancreatic cancer. Pancreatic cancer cells were harvested from subconfluent cultures by brief exposure to 0.25% trypsin (with 0.02% EDTA in PBS). Trypsinization was stopped by adding DMEM medium containing 10% FBS. The cells were washed once in serum-free medium and resuspended in Hank’s balanced salt solution. Single-cell suspensions containing 1x10^6 cells with >90% viability (as determined by trypan-blue exclusion) were injected into the pancreas. After 6 weeks, the mice were euthanized and pancreatic tumors were harvested. A portion of formalin and another portion was immediately embedded in ornithine carbamyl transferase compound, rapidly frozen in liquid nitrogen, and stored at -80˚C.

Statistical analysis. Statistical analysis was done using SPSS software for Windows version 10.0. Correlation analysis was conducted by Spearman's rank correlation test. A P<0.05 was considered statistically significant.

Results

Fibrillar Tn-C matrix formation was increased in human pancreatic liver metastases. To determine the relationship between liver metastasis of pancreatic cancer and the organization of the ECM, we analyzed the expression of Tn-C in 10 metastatic pancreatic cancers and 10 non-metastatic pancreatic cancers. As shown in Table I, the expression of Tn-C was increased in all pancreatic cancers compared to normal pancreatic tissues. In contrast, fibrillar Tn-C (fTn-C) was found only in metastatic pancreatic cancers, but not in non-metastatic pancreatic cancers (Fig. 1). Statistical analysis showed that fTn-C expression is associated with liver metastases of pancreatic cancers (Table II), suggesting that fTn-C matrix formation may play a role in promoting pancreatic cancer metastasis. Since MMP-2 is activated in metastatic pancreatic cancers, we next analyzed the relationship between...
Figure 1. Tn-C matrix formation increased in liver metastasis of human pancreatic cancer. Immunoperoxidase staining of pancreatic adenocarcinomas (case 5 compared to case 6) with anti-Tn-C. The non-metastatic sample (case 5; A) has only moderate Tn-C staining, while the metastatic sample (case 6; B) has intense staining in the stroma. Magnification x100.

Figure 2. Tn-C organization required participation of stromal fibroblast and active MMP-2. Cells were cultured on glass coverslips at 37°C for 48 h. The cells were processed for immunofluorescent staining as described in Materials and methods. The bar represents 50 μm, except in panel 2, where it is 25 μm. (A) Tn-C accumulated on the surfaces of BxPc3 (left) and PaCa2 (right) cells. (B). Tn-C assembled in the co-cultures of BxPc3 cells with stromal fibroblasts. The cells were stained for Tn-C (FITC; green) and FN (Rho; red) in the BxPc3/SF cocultures (panels 1 and 2), the PaCa2/SF cocultures (panel 3), and the BxPc3/NF cocultures (panel 4).
MMP-2 activation and fibrillar Tn-C. MMP-2 activation was associated with fTn-C formation, indicating that MMP-2 may contribute to Tn-C organization.

Fibrillar Tn-C organization required stromal fibroblast participation. To explore the mechanism of Tn-C organization, a co-culture was used to determine whether Tn-C matrix formation required a direct cell-cell interaction. Two pancreatic cancer cell lines were co-cultured with either SF or NF, and Tn-C organization was assessed by immunofluorescence. As shown in Fig. 2A, while both pancreatic cancer cells expressed Tn-C, they were not able to organize fibrillar Tn-C. However, when the metastatic BxPc3 pancreatic cancer cells were co-cultured with SF, fTn-C was found surrounding the cancer cells and colocalized with FN (Fig. 2B, panels 1 and 2), but not in PaCa2/SF co-cultures (Fig. 2B, panel 3) or BxPc3/NF co-cultures (Fig. 2B, panel 4). These results demonstrate that fTn-C organization only occurs when the metastatic BxPc3 pancreatic cancer cells are co-cultured with SF.

Fibrillar Tn-C organization in Bxpc3/SF co-culture required active MMP-2. We have previously shown that MMP-2, rather than MMP-9, is activated in the co-culture of pancreatic cancer BxPc3 cells with SF (3). To explore further the role of MMP-2 in Tn-C organization, we added the MMP-2 inhibitor GM6001 to the BxPc3/SF co-culture. GM6001 suppressed MMP-2 activation and Tn-C organization (Fig. 2C, panel 5).

Table II. Statistical analysis for liver metastasis of pancreatic cancer and the expressions of Tn-C and MMP-2.

<table>
<thead>
<tr>
<th>Liver met.</th>
<th>Tn-C</th>
<th>MMP-2</th>
<th>fTn-C</th>
<th>fTn-C vs MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient</td>
<td>0.345</td>
<td>0.667</td>
<td>0.743</td>
<td>0.575</td>
</tr>
<tr>
<td>Significance</td>
<td>0.137</td>
<td>0.000</td>
<td>0.001</td>
<td>0.008</td>
</tr>
</tbody>
</table>

met, metastasis.
Next, because MMP-2 is not activated in PaCa2/SF co-cultures, we added active MMP-2 into the PaCa2/SF co-culture to observe Tn-C organization. Exogenous MMP-2 promoted the organization of Tn-C in the PaCa2/SF co-culture (Fig. 2C, panel 6), but not to the same degree as seen in the BxPc3/SF co-culture. These results demonstrate that Tn-C organization requires active MMP-2.

To identify how modification by MMP-2 affects Tn-C storage into the pericellular matrix, we compared Tn-C isoforms in the pericellular matrix of the cancer cells with those co-cultured in the presence of the MMP2 inhibitor GM6001. As shown in Fig. 2D, 250, 190 and 120 kDa bands of Tn-C were observed in the BxPc3/SF co-culture. However, only the 250 and 190 kDa isoforms of Tn-C were found in PaCa2/SF co-culture, BxPc3/NF co-culture, pancreatic cancer cells, and BxPc3/SF co-cultured in presence of GM6001. These results suggest that Tn-C storage into ECM requires cleavage by MMP-2, producing a 120 kDa isoform.

Fibrillar Tn-C organization decreased adhesion and promoted migration of pancreatic cancer cells. To evaluate the role of fTn-C in promoting pancreatic cancer cell movement, we tested the effect of fTn-C on cell adhesion and migration. First, the fTn-C from the matrix and non-fibrillar Tn-C from the cancer cells was purified as described in Materials and methods. Since we previously showed that expression of integrin α6β1, an Ln-1 receptor, was up-regulated in BxPc3 cells, we investigated the effect of purified Tn-C on the adhesion of pancreatic cancer cells to Ln-1. As shown in Fig. 3A, the two cell lines showed similar adhesiveness to FN and Col 1, ECM proteins that are not ligands for α6β1. The BxPc3 cells bound to Ln-1 more efficiently than the PaCa-2 cells. When fTn-C was combined with Ln-1, the adhesion of BxPc3 cells to Ln-1 decreased significantly. In contrast, when the non-fibrillar Tn-C was combined with Ln-1, the adhesion of BxPc3 cells to Ln-1 decreased only slightly. Both fTn-C and non-fibrillar Tn-C had little effect on the adhesion of PaCa2 cells to Ln-1. These results demonstrate that fTn-C decreases the adhesion of integrin α6β1 to Ln-1 in the highly metastatic BxPc3 cells, but not in the non-metastatic PaCa2 cells.

We next tested the effect of fTn-C on cell migration (Fig. 3B). When the complex of fTn-C with laminin-1 was used to initiate haptotaxis, migration was augmented greatly in the BxPc3 cells but not in the PaCa-2 cells. In contrast, when the complex of Tn-C with laminin-1 was used to initiate haptotaxis, the migration was not increased in the BxPc3 or the PaCa2 cells. Anti-α6 antibody (GoH3) completely suppressed the migration of BxPc3 cells on the complex of fTn-C with laminin-1. These results indicate that modification of Tn-C by MMP-2 has more potential to promote pancreatic cancer cell migration on Ln-1 by interacting with α6β1.

fTn-C formation is increased in Bxpc3 tumors. To clarify further the relationship between Tn-C formation and active MMP-2 in vivo, we injected pancreatic cancer cells into nude mice and then evaluated fTn-C formation by immunohistochemistry, and MMP-2 activation by Western blotting and zymograph. As shown in Fig. 4, fTn-C formation (Fig. 4A) and active MMP-2 (Fig. 4B) were found in BxPc3 tumors, but not in PaCa2 tumors. These results further confirm our previous observation that Tn-C organization requires MMP-2 activation.

Discussion

In this study, we investigated the role of SF in promoting Tn-C organization in pancreatic cancer. We found that fTn-C increased in metastatic pancreatic cancer. The fTn-C expression correlated with the expression of active MMP-2. In the in vitro co-culture, fTn-C organization was found only in the co-culture of SF with the highly metastatic pancreatic cancer cell line BxPc3. This organization required the participation of SF and active MMP-2. The active MMP-2 could release or modify membrane-bound Tn-C, promoting Tn-C incorporation into the ECM. The fTn-C ECM promoted pancreatic cancer cell movement by decreasing cell adhesion and promoting cell migration.

The stroma surrounding malignant cells differs from normal ECM. A recent study has shown that the ECM protein
Tn-C is expressed in many tumor types and associated with tumor progression (12-15). In our study, we found that Tn-C was abundant in the stroma of metastatic pancreatic cancers but not in non-metastatic pancreatic cancers. Statistical analysis showed that fTn-C expression is associated with liver metastases of pancreatic cancers. These findings are in accordance with the reports from Midwood and co-workers (16,17). Moreover, in these metastatic cancers, fTn-C formation was associated with MMP-2 activation, suggesting that MMP-2 may contribute to Tn-C organization.

Both tumor and stromal cells secrete matrix degrading enzymes, including MMPs that can cleave Tn-C and promote its deposition into the ECM. MMPs are the main physiologically relevant mediators of matrix degradation (18-21). Our results showed that the major Tn-C fragments in pancreatic cancer BxPc3/SF coculture are 190 and 120 kDa. In this coculture, MMP-2, but not MMP-9, is activated (8). These results are consistent with previous reports (11), demonstrating that a large Tn-C variant can be cleaved in vitro into two major fragments of 190 and 120 kDa by MMP-2. Our results suggest that elevated MMP-2 activity not only degrades Tn-C into smaller isoforms, but also promotes Tn-C incorporation into the tumor microenvironment.

Deposition of Tn-C into the matrix is positively regulated by MMPs and stromal fibroblasts (20). We found that Tn-C organization required the participation of active MMP-2 and SF. In the in vitro co-culture, fTn-C was detected only in BxPc3/SF co-culture in which MMP-2 was activated, but not in the PaCa2/SF co-culture, or in pancreatic cancer cells cultured alone, in which MMP-2 was not activated. In the BxPc3/SF co-culture, inhibition of active MMP-2 diminished Tn-C organization. Moreover, the in vivo analysis showed that fTn-C formation and active MMP-2 were found only in BxPc3 tumors. These results suggest that Tn-C deposition into the ECM requires participation of active MMP-2 and SF.

In the process of tumor progression, cell migration is a critical step. It is dependent on the profiles of integrin expression and the components of the ECM (22,23). Although Tn-C is highly expressed in the majority of malignant solid tumors, its roles in promoting cancer metastasis is not clear. In this study, we found that fTn-C has a potential role in promoting pancreatic cancer migration. The BxPc3 cells expressed α6β1 as the major laminin receptor, and adhered and migrated very well on Ln-1. However, addition of fTn-C to Ln-1 matrices augmented the migration of BxPc3 cells by decreasing cell adhesion to Ln-1, but non-fibrillar Tn-C had little anti-adhesion or migration-promoting effect. The increased migration was suppressed by the integrin α6β1 function-blocking antibody in BxPc3 cells. Thus, fTn-C promotes cell migration by decreasing the adhesive abilities of integrin α6β1.

In conclusion, our findings elucidate a metastatic mechanism of pancreatic cancer; that is, during the processes of migration of pancreatic cancer, the expression of integrin α6β1 corresponds to acquisition of the metastatic phenotype of pancreatic cancer cells, thus facilitating cell migration out of the primary tumor. On the other hand, Tn-C, secreted by pancreatic cancer cells, is modified by MMP-2 and deposited into ECM and promotes cell motility in an integrin α6β1-dependent manner. Thus, the deposition of Tn-C into the
ECM in metastatic pancreatic cancer cells creates a permissive environment for tumor cell migration.

Acknowledgments

We thank Dr Guo-dong Liu for editing the manuscript. This work was supported by National Natural Science Foundation of China (30371587), Military Medical Science Foundation of China (06H032).

References