Enhanced Peritoneal Ovarian Tumor Dissemination by Tissue Transglutaminase

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Abstract

Tissue transglutaminase (TG2) is involved in Ca2+-dependent aggregation and polymerization of proteins. We previously reported that TG2 mRNA is up-regulated in epithelial ovarian cancer (EOC) cells compared with normal ovarian epithelium. Here, we show overexpression of the TG2 protein in ovarian cancer cells and tumors and its secretion in ascites fluid and define its role in EOC. By stable knockdown and overexpression, we show that TG2 enhances EOC cell adhesion to fibronectin and directional cell migration. This phenotype is preserved in vivo, where the pattern of tumor dissemination in the peritoneal space is dependent on TG2 expression levels. TG2 knockdown diminishes dissemination of tumors on the peritoneal surface and mesentery in an i.p. ovarian xenograft model. This phenotype is associated with deficient $\beta_1$ integrin-fibronectin interaction, leading to weaker anchorage of cancer cells to the peritoneal matrix. Highly expressed in ovarian tumors, TG2 facilitates i.p. tumor dissemination by enhancing cell adhesion to the extracellular matrix and modulating $\beta_1$ integrin subunit expression. [Cancer Res 2007;67(15):7194–202]

Introduction

Epithelial ovarian cancer (EOC) arises from the epithelial layer covering the surface of the ovaries, and i.p. metastasis is commonly observed at diagnosis. Ovarian tumor spread in the i.p. space leads to the characteristic symptoms and complications of the disease, ascites, and small bowel obstruction. Several features set apart ovarian cancer spread from the metastatic model of other epithelial tumors. First, EOC cells are in direct contact with the overlying peritoneal fluid and this allows exfoliated cells to disseminate freely in the i.p. space. Second, ovarian cancer cells derived from the mullerian epithelium have dual epithelial and mesenchymal characteristics and can convert to either phenotype in response to factors in the microenvironment (1, 2). Adopting a mesenchymal phenotype favors dislodgement from the primary tumor, as cells are more motile and not bound by tight cellular junctions. Thus, EOC cells can spread passively to distant sites by exfoliating from the primary tumor, floating in the peritoneal fluid, and nesting along the i.p. space, where they adhere and grow as metastatic implants. This type of spread, which is uniquely characteristic to EOC, is accompanied by specific changes at the interface between tumor and the peritoneal “oncomatrix” that allow cancer cells to move, attach, and grow.

Such changes include increased expression of integrins (3–6) and of the hyaluronan receptor CD44 (7) that promote adhesion of EOC cells to the peritoneum and overexpression of the chemokine receptor CXCR4 and secretion of its ligand CXCL12 that regulate cell motility in the i.p. milieu (8). Cancer and mesothelial cells secrete lysophosphatidic acid (9) and other proteins [fibronectin, peristatin, osteopontin, and laminin (10–13)], which stabilize the extracellular matrix (ECM) and promote establishment of metastases. These interactions with the mesothelium and the peritoneal stroma activate "outside-in" signaling (14), which stimulates cancer cell proliferation and survival. In this context, neovascularization is facilitated and peritoneal metastases form and grow.

Tissue transglutaminase (TG2) is a ubiquitously expressed enzyme involved in protein cross-linking via acyl transfer between glutamine and lysine residues. TG2 promotes Ca2+-dependent posttranslational protein modification effected by the insertion of isopeptide bonds and incorporation of polyamines into peptide chains. We previously reported that TG2 mRNA expression is up-regulated in transformed ovarian epithelial cells and tumors compared with normal ovarian surface epithelial cells (15). Other reports link TG2 to epithelial cancers, particularly breast (16), pancreatic (17), and non–small cell lung cancer (18). In breast cancer cells, membrane-bound TG2 has kinase activity and phosphorylates the insulin-like growth factor-binding protein-3 (19) and the enzyme is overexpressed in drug- and radiation-resistant breast cancer cells (20, 21). The goal of this study was to analyze the role of TG2 as a mediator of ovarian tumor dissemination in the peritoneal space.

We show here that TG2 is expressed in a cancer-specific manner in human ovarian tumors and is secreted in ascites fluid. We show that TG2 facilitates ovarian cancer cell adhesion to fibronectin and directional cell migration and promotes i.p. tumor seeding. TG2 exerts its effects by interacting with $\beta_1$ integrin, modulating its expression and function. These data suggest a novel role for TG2 as a regulator of i.p. metastasis.

Materials and Methods

Immunohistochemistry. Twenty-seven paraffin-embedded epithelial ovarian tumor specimens from the Cooperative Human Tissue Collection and 6 normal ovarian specimens from six patients undergoing oophorectomy for benign disorders from the Indiana University Tissue Bank Collection were immunostained using a TG2 monoclonal antibody (CUB 7402, NeoMarkers) at a dilution of 1:200 after antigen retrieval using sodium citrate. Secondary labeling was based on the avidin/biotin system (LSAB2 kit, DAKO). Slides were stained with 3,3′-diaminobenzidine and counterstained with hematoxylin. Negative controls were run in parallel, with omission of the primary antibody. Staining was graded from 0 (no staining) to 4 (maximal staining).
to 3+ (strong staining) by a board-certified pathologist. Immunoreactivity was recorded only if noted in more than 15% to 20% of tumor cells. The Indiana University Institutional Review Board approved the use of human tissue specimens (protocol 0412-54).

**Ascites fluid.** Thirty samples of ascites fluid cytologically positive from patients with ovarian cancer and 8 samples of ascites fluid from patients with noncancerous conditions (inflammatory pleural or ascitic fluid) were included in this analysis (University of California at Los Angeles and Indiana University Cancer Center Tissue Bank, protocol collection approved by the Institutional Review Board, protocol 0409-02). After collection, samples were centrifuged to remove cellular debris, aliquoted, and stored at −80°C until use.

**Cell lines.** Human SKOV3 and OV90 ovarian cancer cell lines were obtained from the American Type Culture Collection and cultured in growth medium containing 1× MCD-105 (Sigma) and M199 (Cellgro) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cellgro) and 1% antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). All cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

**Transfection.** To overexpress TG2, OV90 cells in the logarithmic phase of growth were transfected with wild-type TG2 cloned into the pcdNA3.1 vector using Fugene (Roche Applied Science). To knock down TG2, an antisense construct cloned into pcdNA3.1 vector was transfected in SKOV3 cells. As a control, cells were transfected with the pcdNA3.1 vector carrying the G418 resistance gene. Transfection efficiency in these conditions is typically 5% to 10% in OV90 cells and 30% to 40% in SKOV3 cells as determined by estimation of green fluorescent protein expression. Stable transfected clones were established by selection with G418 (Sigma) at 400 µg/mL, 100 µg/mL paraformaldehyde, cells were permeabilized using Triton X-100 (0.2% in PBS containing 1 mmol/L MgCl₂, 10 mmol/L Tris-HCl (pH 7.4), and 2 mmol/L PMSF. The cell lysate served as a negative control. Nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 20 min. After washing, cells were resuspended in serum-free medium. Equal numbers of cells (4 × 10⁵ per well) were seeded into 96-well plates precoated with fibronectin (Sigma) at different concentrations (1–10 µg/mL) or bovine serum albumin (1% w/v). After 1 h of incubation at 37°C, the plate was immersed into PBS containing 1 mmol/L MgCl₂ to remove nonadherent cells. The number of adherent cells was measured in a fluorescence plate reader (Applied Biosystems) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All experiments were done in quadruplicate and repeated twice.

**Cell migration assays.** A migration assay was done in a modified Boyden chamber method using 6.5-mm-diameter, 8.0-µm pore size polycarbonate membrane transwell inserts in a 24-well plate (Corning). To assess directional migration, the lower surfaces of the transwell polycarbonate membrane transwell inserts in a 24-well plate (Corning). To assess directional migration, the lower surfaces of the transwell were stained with HEMA3 stain (Fisher) and counted at ×200 magnification. Cells were counted in five high-power fields (HPF) in duplicate experiments. Results are expressed as mean number of migrating cells ± SE. A similar experiment was done using serum-free conditioned medium from cells stably transfected with AS-TG2 or pcdNA3.1 as cell attractant in the lower chamber of the transwell.
In vivo growth of SKOV3 cells in nude mice. The human ovarian cancer cell line SKOV3 stably transfected with AS-TG2 or vector was injected i.p. into 7- to 8-week-old female nude mice (nu/nu BALB/c) from Harlan. Eight weeks after the injection, the mice were euthanized and a necropsy was done. Tumor formation was estimated by two methods. First, we measured bidimensionally tumors >0.4 cm with calipers and calculated tumor volume according to the formula \( \frac{L \times W^2}{2} \), where \( L \) is length and \( W \) is width. A cumulative tumor volume was calculated by adding the volumes of dominant tumors for each animal. Second, we estimated peritoneal seeding with unmeasurable 1- to 3-mm tumors by counting the number of implants on the mesentery, omentum, and peritoneum. Harvested tumors were preserved in formalin for future histologic and immunohistochemical studies. When possible, tumors were minced and cultured in medium supplemented with G418 in the presence of hyaluronidase at a concentration of 1 mg/mL. The xenograft-derived cultures were characterized by immunoblotting and solid-phase assay. Animal experiments were approved by the Indiana University School of Medicine Animal Care and Use Committee (protocol 2680) and were in accordance with federal regulations. Three independent experiments were done.

Flow cytometry. Quantification of cell surface \( \beta_1 \) integrin was done using the FACScan/CellQuest system (Becton Dickinson). Trypsinized cells were incubated with \( \beta_1 \) integrin monoclonal antibody (1:100 dilution) or mouse IgG for 1 h on ice. After incubation with secondary AlexaFluor488-labeled anti-mouse IgG (1:500 dilution), immunofluorescent staining was quantified using the FACScan/CellQuest system. Ten thousand events were accumulated for each analysis. Three independent readings were obtained from separate experiments, and data were averaged for statistical analysis.

Statistical analysis. For the analysis of the immunohistochemical and immunoblotting data in cancer and noncancer specimens, the \( \chi^2 \) test was used. Likewise, the \( \chi^2 \) was used for the comparison between animals developing peritoneal seeding in the groups injected with AS-TG2–transfected or control cells. For the solid-phase adhesion and migration assays, flow cytometry analysis, and comparison of volumes and number of peritoneal implants between the two animal groups, we used the Student’s \( t \) test.

Results

TG2 is expressed in EOC. We used immunohistochemical assay to determine expression of TG2 in ovarian tumors. Among 27 tumors, we found intense cytoplasmic and membrane staining (2 to 3+) in 21 specimens (79% of tumors; Fig. 1A). Two specimens stained weakly (1+) and four tumors did not stain. All histologic subtypes were immunoreactive: 9 of 9 clear cell carcinoma were intensely positive, 3 of 3 endometrioid tumors displayed 2+ staining, and 9 of 14 papillary serous tumors were 2 to 3+ positive. One poorly differentiated carcinoma did not immunoreact. Details of tumor characteristics, including stage, histologic type, and grade, are included in Supplementary Table S1. TG2 expression was noted in advanced tumors (10 of 13 stage III and IV tumors) as well as in early-stage disease (13 of 14 stage I and II tumors). In normal ovary, TG2 immunoreactivity was weak in normal stroma and absent in the surface epithelial layer. None of six normal ovarian specimens immunostained for TG2 in the epithelium, suggesting that TG2

Figure 1. A, expression of TG2 by immunohistochemistry in ovarian tumors and normal ovary. 1 and 2, normal ovary. Arrows point to normal ovarian epithelium and to a surface epithelial inclusion. 3 and 4, representative ovarian tumors. B, TG2 expression in ascites specimens. Each immunoblot includes six specimens of malignant ascites from patients with EOC (lanes 1–6), two specimens of ascites fluid or pleural fluid from patients with nonmalignant diseases (lanes 7 and 8), and a positive control (lysate from ovarian cancer cell line, SKOV3). Equal volume (30 μL) of ascites fluid was loaded in each lane.
expression is specific to transformed ovarian epithelial cells ($P = 0.005$), but weak staining was observed in surface epithelial inclusions (Fig. 1A). Control staining (without primary antibody) was consistently negative.

**TG2 is secreted in EOC malignant ascites.** Because ovarian cancer disseminates in the peritoneal cavity, large volumes of ascites that contain proteins secreted by tumor and mesothelial cells are generated (22). Such secreted proteins modulate the growth and spread of carcinoma cells in the peritoneal milieu (9, 23, 24). Knowing that TG2 is a secreted protein, we tested whether it is detectable in ascites. Immunoblot analysis revealed the presence of TG2 in 25 of 30 ascites specimens from patients with EOC (representative immunoblots illustrated in Fig. 1B). There was variable level of TG2 secretion among the malignant ascites specimens. Eight specimens of nonmalignant, inflammatory effusions contained none or negligible TG2 (one of eight samples), suggesting that TG2 secretion is specific to cancer cells ($P = 0.005$). Immunoblot analysis revealed a higher molecular weight band, migrating at $\sim 170$ kDa in several ascites specimens. This was also observed in conditioned medium from some of the ovarian cancer cell lines (data not shown) and was partially disrupted by stringent denaturing conditions (SDS or 2-mercaptethanol), suggesting that

![Figure 2.](image.png)

**Figure 2.** Effects of TG2 knockdown on ovarian cancer cell adhesion and migration. A, Western blot analysis for TG2 using cell lysates and conditioned medium. G and M are stable clones identified by selection with G418 after transfection with AS-TG2. Controls are untransfected cells (UT) and cells stably transfected with pcDNA3.1. Serum-free conditioned medium from AS-TG2 clone (G) was compared with conditioned medium from pcDNA3.1-transfected cells by immunoblotting. Equal volume of conditioned medium was loaded in each lane (30 μL). B, solid-phase assay measured adhesion to fibronectin (FN) for vector-transfected and AS-TG2–transfected cells. Number of cells adhering to fibronectin within 1 h was quantified based on fluorescence emission after cells were labeled with calcein AM [relative fluorescence units (RFU)]. Points, mean of four replicates; bars, SE. $P = 0.006$. BSA, bovine serum albumin. C, staining for phalloidin in SKOV3 cells stably transfected with AS-TG2 or vector. Cells were plated on fibronectin-coated coverslips, allowed to adhere for 60 min, and then fixed and stained with rhodamine-phalloidin antibody. Red signal, visualization was done under UV excitation at 520 nm with a confocal microscope. D, effects of TG2 on directional cell migration. Left, directional migration stimulated by collagen or fibronectin as measured in SKOV3 cells stably transfected with AS-TG2 or pcDNA3.1 by transwell assay. Cells ($1 \times 10^6$) were plated in each well. Cells migrating to the lower surface of the filter within 5 h were counted. Columns, average of cells counted per 10 HPF for each experimental condition; bars, SE. $P < 0.001$. Right, directional migration stimulated by fibronectin and conditioned medium (CM) in SKOV3 cells. SKOV3 cells ($1 \times 10^6$) were plated in each well. In the lower chamber, the medium consisted of serum-free medium (negative control; first column), medium with 20% FBS (positive control; second column), and serum-free conditioned medium collected from SKOV3 cells stably transfected with AS-TG2 (third column) or pcDNA3.1 (fourth column). Cells migrating to the lower surface of the filter within 5 h were counted. Columns, average of cells counted per 10 HPF; bars, SE. $P < 0.001$. 

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it represents a covalently linked dimer. These data suggest that TG2 is up-regulated in EOC cells and secreted in malignant ascites.

**TG2 facilitates ovarian cancer cell adhesion to fibronectin and directional cell migration.** To understand the function of TG2 in ovarian cancer cells, we generated stable human cell lines, in which TG2 was overexpressed or knocked down. To knock down TG2, we used an antisense construct (AS-TG2) cloned in pcDNA3.1 in which TG2 was overexpressed or knocked down. To knock down TG2 in ovarian cancer cells, we generated stable human cell lines, and directional cell migration.

Next, we wished to determine whether TG2 mediated ovarian cancer cell dissemination in the peritoneal space and examined its involvement in cell adhesion and directional migration. Using a solid-phase assay, we found that stable knockdown of TG2 in SKOV3 cells decreased ovarian cancer cell adhesion to fibronectin by >50% at all concentrations of fibronectin tested compared with control cells (Fig. 2B). Cell spread on fibronectin was visualized by staining the cytoskeleton with rhodamine-phalloidin. AS-TG2-transfected cells were round and failed to extend lamellipodia, whereas cells transfected with vector spread readily on fibronectin (Fig. 2C).

The effects of TG2 on directional cell migration were measured by a transwell assay. Fibronectin- and collagen-stimulated chemotaxis was decreased in SKOV3 cells stably transfected with AS-TG2 compared with cells transfected with vector (Fig. 2D). In addition, secreted TG2 acted as an attractant, stimulating ovarian cancer cell migration across the transwell. Directional cell migration of SKOV3 cells induced by conditioned medium was significantly lower when the assay was done with conditioned medium lacking TG2 (from AS-TG2 cells) compared with conditioned medium from control cells (Fig. 2D).

Next, we tested whether stable overexpression of TG2 increases adhesion to fibronectin. For this, an ovarian cancer cell line with low endogenous TG2 (OV90) was transfected with TG2. A stably transfected clone was identified by immunoblotting after selection with G418 (Fig. 3A). Stable expression of TG2 enhanced adhesion to fibronectin compared with cells transfected with empty vector (Fig. 3B). Likewise, directional cell migration stimulated by fibronectin or collagen was enhanced by stable expression of TG2 (Fig. 3C). Coupled with the effects of TG2 knockdown, these experiments show that TG2 is critical to ovarian cancer cell adhesion and directional migration, essential steps in metastasis.

**TG2 knockdown inhibits tumor development and spread on the peritoneal surface in vivo.** We injected nude mice i.p. with SKOV3 cells stably transfected with AS-TG2 or empty vector, and after 8 weeks, we observed a significant difference in pattern of tumor development. Mice injected with pcDNA3.1-transfected (empty vector) cells developed tumors in the omentum and the retroperitoneal space and numerous 1- to 3-mm tumors studding the peritoneal surface adjacent to the bowel and on the peritoneal surface of abdominal flanks (Fig. 4A and B). The average number of

**Table 1. Tumor formation in the peritoneal space according to TG2 expression**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. mice</th>
<th>pDNA3.1</th>
<th>AS-TG2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>5</td>
<td>6</td>
<td>268 ± 33 214 ± 40</td>
<td>0.392</td>
</tr>
<tr>
<td></td>
<td>81 ± 11</td>
<td>10.5 ± 4.3</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>5</td>
<td>5</td>
<td>172 ± 70 342 ± 90</td>
<td>0.392</td>
</tr>
<tr>
<td></td>
<td>65.8 ± 12</td>
<td>14.6 ± 5.4</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Experiment 3</td>
<td>4</td>
<td>6</td>
<td>1152 ± 394 369 ± 83</td>
<td>0.139</td>
</tr>
<tr>
<td></td>
<td>81.5 ± 23</td>
<td>24 ± 18</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>14</td>
<td>17</td>
<td>486 ± 130 306 ± 44</td>
<td>0.288</td>
</tr>
<tr>
<td></td>
<td>76 ± 8</td>
<td>17 ± 6</td>
<td>0.00001</td>
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</tbody>
</table>

**NOTE:** Data are presented as average volume and average number of implants ± SE.

* Tumor volume was calculated by adding the volume of individual tumors >0.4 cm in greatest dimension for each animal.

† Statistically significant.
The tumor volume of dominant masses was not significantly different for AS-TG2–derived xenografts compared with controls (Table 1). Twelve of 14 mice injected with control cells developed milliary peritoneal metastases, whereas only 2 of 17 mice injected with

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**Figure 4.** TG2 knockdown inhibits tumor development and i.p. dissemination in vivo. **A,** in vivo tumor development. Nude mice injected with control cells (pcDNA3.1-SKOV3) form tumors studding the mesentery and peritoneal surface as well as tumors infiltrating the retroperitoneal space. Mice injected with AS-TG2–transfected cells form tumors in the retroperitoneal space, at the site of the i.p. injection, and have clear mesentery. The images of open animals provide a direct view of the small bowel and mesentery. In the animal injected with pcDNA3.1-transfected cells, arrow points to large tumors on the mesentery adjacent to the small bowel. In the mouse injected with AS-TG2 cells, arrow points to tumor nodule at the site of i.p. injection. Macroscopically, the bowel and mesentery appear clear. Pieces of small bowel and adjacent mesentery were photographed at × 12 magnification with a SteREO SV11 Apo Zeiss dissecting microscope. In the mouse injected with AS-TG2 cells, the arrow points to clear mesentery. In the animal injected with control cells, the multiple arrows indicate many tumor implants studding the mesentery. **B,** histologic appearance of xenografts (H&E staining). 1, no tumor is visualized on the mesentery in animals injected with AS-TG2 cells; 2 and 3, block of tumor derived from pcDNA3.1-SKOV3 cells invading the mesentery adjacent to bowel; 2 and 4, AS-TG2–derived and pcDNA3.1-derived tumor infiltrating the pancreas. Arrows point to tumor deposits in sections 2, 3, and 4. In section 1, arrow points to clear mesentery adjacent to normal bowel. **C,** expression of TG2 by immunohistochemistry in xenografts. 1, negative control (no primary antibody, pcDNA3.1-derived tumor); 2, TG2 staining is absent in tumors derived from AS-TG2 cells; 3 and 4, intense TG2 staining noted in tumors derived from pcDNA3.1-transfected SKOV3 cells. 3, arrow, a peritoneal implant in the mesentery adjacent to normal bowel. **D,** characteristics of xenograft-derived cell cultures. Expression of TG2 and β1 integrin assessed by immunoblotting is diminished in cultures derived from AS-TG2 xenografts compared with controls (pcDNA3.1 xenografts). Adhesion to fibronectin, as measured by solid-phase assay, is also diminished in cells derived from AS-TG2 xenografts compared with controls. Fold difference in measured fluorescence (relative fluorescence units) corresponding to the number of cells adherent within 1 h to fibronectin-coated surfaces. Fibronectin concentrations varied between 1 and 10 μg/mL.

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implants was 76 ± 8. Mice injected with AS-TG2–transfected cells developed one large tumor in the omentum, invading into the retroperitoneal space, and a tumor nodule at the injection site, but significantly fewer mesenteric implants (17 ± 6; Table 1). The
AS-TG2 cells developed milliary studding of the mesentery ($P < 0.0001$; Supplementary Table S2). Tumors were of similar histologic appearance, with high nuclear grade for both groups, and a serous papillary pattern was discernible in mesenteric implants.

TG2 knockdown was retained in vivo as shown by immunohistochemistry in xenografts and by Western blot analysis of cell cultures established from explanted xenografts (Fig. 4C and D). Occasional small and isolated islands of TG2-positive cells were observed in tumors from AS-TG2 cells, consistent with the emergence of some TG2-positive subpopulations in the absence of G418 selection in vivo. However, general preservation of the TG2 knockdown in vivo is shown by the observation that cell cultures from explanted xenografts conserved their original phenotype (Fig. 4D). In addition, decreased adhesion to fibronectin was noted between cells cultured from AS-TG2 and control tumors. The pattern of tumor formation in the peritoneal space was consistent with the phenotype observed in vitro, suggesting an important role for TG2 in peritoneal studding.

**TG2 interacts with $\beta_1$ integrin and modulates its expression.**

As alteration in the level of TG2 expression modulates EOC cell adhesion in vitro and in vivo, we examined whether TG2 interacts with integrins. Knowing that $\beta_1$ integrin has been linked to invasion and metastasis in EOC (26), we evaluated interaction of TG2 with $\beta_1$ integrin. Immunoprecipitation with $\beta_1$ integrin antibody followed by immunoblotting for TG2 shows endogenous interaction between TG2 and $\beta_1$ integrin in EOC cells. TG2 and $\beta_1$ integrin also colocalized in cytoplasmic organelles (Fig. 5A). We also examined the level of $\beta_1$ integrin in cells with diminished TG2 expression and found that the $\beta_1$ subunit is expressed at decreased levels in AS-TG2 transfected cells (Fig. 5B). However, mRNA levels were not different between AS-TG2 and control cells, suggesting that TG2 affects integrin processing posttranscriptionally. Decreased $\beta_1$ subunit in the plasma membrane fraction was confirmed by Western blotting in SKOV3 transfected with AS-TG2 compared with controls. Expression of $\beta_1$ integrin on the cell surface was estimated by fluorescence-activated cell sorting (FACS) and immunofluorescent staining. FACS analysis revealed reduced levels of $\beta_1$ integrin on the surface of AS-TG2 cells compared with controls (Fig. 5C).

In cells expressing AS-TG2, immunofluorescence also showed reduced distribution of $\beta_1$ integrin to the cell membrane (Fig. 5D).

To measure whether changes in rates of protein degradation contribute to the difference in $\beta_1$ integrin expression dependent on the TG2 status of ovarian cancer cells, we treated AS-TG2 and control cells with cycloheximide and measured $\beta_1$ integrin levels by immunoblotting. In AS-TG2 cells, $\beta_1$ integrin was degraded more rapidly [half-life ($t_{1/2}$) = 3–6 h] compared with control cells ($t_{1/2}$ = 12–24 h; Supplementary Fig. S1). To assess lysosomal-, ubiquitin-, or calpain-mediated $\beta_1$ integrin proteolysis in AS-TG2 or pcDNA3.1-transfected (vector) ovarian cancer cells, we measured integrin levels by immunoblotting after incubating cells

![Image](cancerrease.aacjournals.org)
with lysosomal inhibitor (leupeptin), proteasomal inhibitors (MG132 and lactacystin), calpain inhibitor (PD105606), and protein trafficking inhibitors (monensin and chloroquine). Treatment with the proteasomal inhibitor MG132, and more prominently with the calpain inhibitor PD105606, restored β1 integrin levels in AS-TG2 cells (Supplementary Fig. S1B–D), suggesting that TG2 interferes with calpain-mediated β1 integrin degradation in EOC cells. In contrast, inhibition of lysosomal proteases (leupeptin) did not significantly alter integrin levels in AS-TG2 cells (Supplementary Fig. S1E). These observations may explain the selective down-regulation of β1 integrin levels in cells transfected with AS-TG2 and identify a novel function of TG2 in epithelial cancer cells.

Discussion

I.p. metastasis characteristic of EOC requires modifications of tumor cells to facilitate interaction with the peritoneal stroma and mesothelium. In this report, we show overexpression of TG2 in ovarian tumors and its secretion in malignant ascites fluid. We show that TG2 mediates ovarian cancer cell adhesion to fibronectin and stimulates directional cell motility, these processes being mediated by TG2 via interaction and stabilization of β1 integrin. Using an i.p. xenograft model, we show that TG2 knockdown decreases the pattern of diffuse tumor spread, implicating it as a mediator of i.p. metastasis.

Having identified TG2 as an overexpressed transcript in primary EOC cells (15), we show here that >75% of ovarian tumors overexpress the protein. TG2 is not expressed on the surface ovarian epithelium but is present in stage I and II ovarian tumors. TG2 up-regulation has been reported in glioblastoma, pancreatic, breast, and lung cancer, and a multitude of functions has been invoked for it (21, 27, 28). We focus here on the induced stabilization of cell adhesion of TG2, as this is critical to the establishment of i.p. metastases, where cancer cells are required to “stick” to the oncomatrix to establish peritoneal implants. Adhesion to fibronectin and chemotaxis was decreased in EOC cells by knockdown of TG2 and enhanced by stable overexpression of the protein. This phenotype was preserved in vivo, where the pattern of distribution of i.p. implants was altered by TG2 knockdown. Control animals injected with SKOV3-pcDNA3.1 cells developed i.p. implants widely disseminated on the mesentery and few or no mesenteric metastatic foci, suggesting that TG2 plays an important role in mediating the invasiveness of ovarian tumors and clinical outcome (26, 34, 35) as well as sensitivity to chemotherapy (36–38). Integrin signaling has been linked to cancer metastasis (39–41), and disruption of integrin function by competing antibodies or peptides has been proposed as a possible cancer treatment (42–44). The data presented here provide compelling evidence that TG2 may be a target, as its interaction with and regulation of integrins directly promote cell adhesion, which is an important step in peritoneal metastasis.

We also found that TG2 is secreted abundantly in malignant ascites fluid. This is likely due to accumulation from tumor and/ or mesothelial cells, as primary ovarian cancer cells and cancer cell lines secrete TG2. Although TG2 lacks a leader sequence, it is secreted into the extracellular space through an as yet unknown mechanism (45). The expression of TG2 in tumor cells supports its detection in ascites fluid. We cannot exclude the contribution of mesothelial cells to the composition of malignant ascites; however, the absence of TG2 in inflammatory ascites of pleural fluid makes it likely that TG2 is secreted directly by the tumor cells. Other factors critical to i.p. metastasis are secreted abundantly in ascites, fibronectin, lysophosphatidic acid, hyaluran, and vascular endothelial growth factor, making the i.p. milieu favorable for cancer cell growth (11, 46). The presence of TG2 in the peritoneal fluid may play a role in remodeling the oncomatrix by cross-linking of ECM proteins.

The important conclusion of this study is that TG2 is highly expressed in human ovarian tumor cells and is secreted in malignant ascites. At the interface between cancer cells and the stroma, TG2 alters the pattern of tumor growth and dissemination in the peritoneal space by modulating β1 integrin expression and function.

Acknowledgments

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