Clinical and Biological Significance of Tissue Transglutaminase in Ovarian Carcinoma


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Abstract

Tissue type transglutaminase (TG2) is a unique multifunctional protein that plays a role in many steps in the cancer metastatic cascade. Here, we examined the clinical (n = 93 epithelial ovarian cancers) and biological (in vitro adhesion, invasion, and survival and in vivo therapeutic targeting) significance of TG2 in ovarian cancer. The overexpression of TG2 was associated with significantly worse overall patient survival in both univariate and multivariate analyses. Transfection of TG2 into SKOV3ip1 cells promoted attachment and spreading on fibronectin-coated surfaces and increased the in vitro invasive potential of these cells. Conversely, TG2 silencing with small interfering RNA (siRNA) of HeyA8 cells significantly decreased the invasive potential of the cells and also increased docetaxel-induced cell death. In vivo therapy experiments using chemotherapy-sensitive (HeyA8) and chemotherapy-resistant (HeyA8-MDR and RMG2) models showed significant antitumor activity both with TG2 siRNA-1,2-dioleoyl-sn-glycero-3-phosphatidylcholine alone and in combination with docetaxel chemotherapy. This antitumor activity was related to decreased proliferation and angiogenesis and increased tumor cell apoptosis in vivo. Taken together, these findings indicate that TG2 overexpression is an adverse prognostic factor in ovarian carcinoma and TG2 targeting may be an attractive therapeutic approach. [Cancer Res 2008;68(14):5849–58]

Introduction

Based on current cancer statistics, ovarian cancer remains the most common cause of death from a gynecologic malignancy in the United States (1). This poor outcome relates to the fact that most patients present with advanced stage disease and widespread peritoneal metastasis. Whereas most ovarian cancer patients respond to initial therapy of cytoreductive surgery and platinum-based chemotherapy, more than 70% will recur and eventually succumb to disease (2). Therefore, understanding the molecular factors responsible for ovarian cancer metastasis and development of novel therapeutic approaches is urgently needed.

Tissue transglutaminase (TG2) is a unique multifunctional protein that can catalyze Ca2+-independent hydrolysis of GTP and ATP, the protein disulfide isomerase reaction (3), and Ca2+-dependent posttranslational modification of proteins (4). TG2 also has serine/threonine kinase activity (5–7). The ability of TG2 to hydrolyze GTP enables it to serve as a signaling molecule, leading to the activation of a cytoplasmic target, phospholipase C (8). Although mainly a cytoplasmic protein, TG2 can also be secreted outside the cell where it regulates cell-matrix interactions (9) and can translocate to the nucleus where it associates with p53, histones to regulate certain cellular functions (10–12). Importantly, TG2 can also exist on the cell surface membrane in association with specific integrin family members (β1, β3, β4, and β5), for which TG2 serves as a coreceptor, and promotes stable interactions between cells and the extracellular matrix (ECM), resulting in increased integrin-mediated cell adhesion, spreading, migration, and survival functions (13, 14). TG2 has been shown to activate the RhoA and mitogen-activated protein kinase pathways, which control key downstream signaling steps that affect the invasive and metastatic behavior of malignant cells (15). TG2 overexpression has been noted in several cancers including malignant melanoma (16), breast cancer (17, 18), and pancreatic cancer (19). In these cancers, TG2 contributes to the development of chemoresistance by exploiting integrin-mediated cell survival signaling pathways. In addition, TG2 overexpression contributes to cancer cell adhesion and invasion. Based on these features, TG2 seems to be an attractive therapeutic target.

We have recently developed a highly efficient in vivo method for systemic delivery of small interfering RNA (siRNA) by using a neutral liposome, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC; ref. 20). Our proof-of-concept studies with targeting EphA2 (20) or focal adhesion kinase (FAK; ref. 21) showed the therapeutic efficacy of this approach. In the current article, we show the clinical significance of TG2 as a prognostic factor for ovarian cancer patients. In addition, we characterize the biological effects of TG2 on ovarian cancer progression and use a therapeutically relevant approach for TG2 silencing.

Materials and Methods

Ovarian cell lines and culture condition. The ovarian cancer cell lines HeyA8, SKOV3ip1 (20, 21), A2780-PAR, A2780-CP20, OVCA2, IGROV1, ES2, 222, and RMG2 were cultured in RPMI 1640 supplemented with 10% to 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts). The taxane-resistant HeyA8-MDR (a kind gift from Dr. Isaiah Fidler, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX) and SKOV3-TR cells were maintained in

Note: Supplementary data for this article are available at Cancer Research Online. (http://cancerres.aacrjournals.org/). J.Y. Hwang, L.S. Mangala, and J.Y. Fok contributed equally to this work. K. Mehta and A.K. Sood share senior authorship for this article. Requests for reprints: Anil K. Sood, Departments of Gynecologic Oncology and Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Unit 1362, 1155 Herman Pressler, Houston, TX 77030. Phone: 713-745-5266; Fax: 713-792-7586; E-mail: assood@mdanderson.org. ©2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-6130
by two researchers and one pathologist. The scoring was done based on staining intensity (1, low; 2, moderate; 3, high) and the percentage of positive cancer cells (0, ≤5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, ≥76%). A composite score was generated as the sum of these two variables and the expression was dichotomized as TG2 overexpression (overall score >4) or low/absent expression (overall score ≤4). The χ2 test was used to determine differences among variables using the Statistical Package for the Social Sciences (SPSS, Inc.). Kaplan-Meier survival plots were generated, and comparisons between survival curves were made with the log-rank statistic. The Cox proportional hazards model was used for multivariate analysis. P < 0.05 was considered statistically significant.

Wild-type and C277S mutant TG2 adenovirus generation. An adenovirus containing full-length TG2 (TG2-W) or C277S mutant (TG2-M) cDNA was kindly provided by Dr. Ugra Singh (The Texas A&M University System Health Science Center, Temple, TX). TG2 cDNA cloned in pCDNA3.1 vector was first subcloned in a phushte 2 vector and then in a BD adenoX adenoviral vector. Human embryonic kidney (HEK293) cells were transfected with recombinant adenoviral plasmid for packaging of adenovirus particles followed by purification of adenovirus on CsCl gradient and used at 25 multiplicities of infection. Cells that were infected with lacZ adenovirus served as control. SKOV3ip1 cells, which had low baseline TG2 expression, were infected with adenovirus alone (empty vector) or TG2-W or TG2-M. After 48 h, TG2 activity and protein expression were determined. Same cells were used for cell attachment, invasion, and survival assays. SKOV3ip1 cells were selected for these experiments because from our experience, we have found that cells with complete lack of TG2 expression are not able to sustain TG2 expression (presumably due to low GTP and/or perturbation of cytosolic free calcium resulting from transfection, leading to cross-linking of proteins and cell death).

Cell attachment, invasion, and survival. For adhesion assays, cells (3 × 10^5 per well per 0.2 mL serum-free RPMI 1640) were incubated in fibronectin- or bovine serum albumin (BSA)—coated 96-well plates. After 1-h incubation at 37°C, nonadherent cells were removed with washing with PBS and attached cells were stained with crystal violet and observed under a microscope for morphologic analysis; absorbance was measured at 540 nm for quantitative analysis. The invasive potential of cells was determined in vitro by using Matrigel transwell inserts as described earlier (25). Briefly, Matrigel transwell inserts of 12-μm pore size were coated with 0.78 mg/mL Matrigel in cold serum-free RPMI 1640. The cell suspension (1 × 10^5 cells) was added to duplicate transwells. After incubation for 48 h, the cells that passed through the filter on the underside of the membrane were stained and counted under a light microscope. Tens fields of cells were counted for each well, and the mean number of cells per field was calculated. The number of viable cells remaining after appropriate treatment was determined by measuring their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTT) into a soluble formazan. The cells were plated on fibronectin-coated 96-well plates. After 12 to 24 h of incubation of cells for attachment, the medium was exchanged with increasing concentrations of drugs (0–25 mL/L of paclitaxel or 0–50 mL/L of docetaxel). After incubation for 72 h in the presence of drugs, cells were incubated with 0.15% MTT for 2 h at 37°C. The supernatant was removed, cells were dissolved in 100 μL DMSO, and the absorbance was recorded at 570 nm. Each experiment was repeated thrice in triplicate.

TG2 down-regulation by siRNA and liposomal siRNA preparation. TG2-targeted specific siRNA sequence (5′-AAGGGCGAACCACCTGGAACA-3′) was synthesized and then purified by high-performance liquid chromatography with Quagen-Xenagen. A sequence (5′-TTTCTTGGAGGTTGACG-3′) that did not have homology to any human mRNA as determined by Blast search served as a control compared with a TG2-targeted sequence. At 70% confluence, HeyA8 cells were transfected with either control siRNA or TG2-specific siRNA as described earlier (17). Briefly, 3 × 10^5 cells were plated in each well of six-well plates and allowed to adhere for 24 h. After the day of transfection, cells were washed, transfected, and harvested at different time intervals. The down-regulation of TG2 gene and protein expression were determined by reverse transcription-PCR and Western blot. For in vitro delivery, siRNA was incorporated into the phospholipid DOPC as previously
described (20). Before in vivo administration, this preparation was hydrated with normal (0.9%) saline at a concentration of 15 μg/mL to achieve the desired dose in a 150- to 200-μL volume per injection.

Generation of orthotopic in vivo model and tissue collection after therapy. Female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center. The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. All studies were approved and supervised by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. The mice used in these experiments were 8 to 12 wk old.

To evaluate the therapeutic effect of combination TG2 siRNA and docetaxel in our mouse model, we first conducted preliminary dose-finding experiments for TG2 siRNA. HeyA8 cells (0.5 × 10⁶) were injected i.p. and treatment was initiated 18 d following tumor cell injection when tumors could be assessed by palpation. Mice (n = 3 per group) were given two injections of TG2 siRNA-DOPC 3.5 μg (105 μg/kg) or 5 μg (150 μg/kg) and the tumor was harvested at various time intervals. The i.p. route for siRNA delivery was selected based on comparable uptake and therapeutic efficacy with either i.p. or i.v. routes in a 200-μL volume (36). For therapy experiments, docetaxel was injected once weekly at 50 μg per mouse based on previous experiments for docetaxel kinetics for HeyA8 cells (21) and similar IC₅₀ levels of docetaxel in both HeyA8 and RMG2 cells (data not shown). Following treatment, the mice were sacrificed at 48 h, 96 h, or 6 d. Mouse weight, tumor weight, number of nodules, and tumor site were recorded. Tissue specimens were snap frozen for lysate preparation, fixed in formalin for paraffin embedding, or frozen in optimum cutting temperature (OCT) compound (Miles, Inc.) for frozen slide preparation. Immunohistochemical and Western blot analyses were done on tissue specimens to determine adequate dosage of TG2 siRNA-DOPC for further in vivo therapy experiments.

Based on the results of our preliminary dose-response experiments, we initiated a series of separate therapy experiments using the optimal TG2 siRNA dosage (50 mice per each cell line and 10 mice per each treatment group were used). Tumor cells (0.25 × 10⁶ HeyA8 or 1.0 × 10⁶ HeyA8-MDR or 3.5 × 10⁶ RMG2 cells per mouse) were injected i.p., and 7 d later, mice were randomly assigned to five treatment groups: empty liposome twice weekly, nonspecific control siRNA-DOPC (150 μg/kg) twice weekly, control siRNA-DOPC in combination with docetaxel, TG2 siRNA-DOPC (150 μg/kg) twice weekly, and TG2 siRNA-DOPC plus docetaxel. Mice were monitored for adverse effects, and tumors were harvested after 3 to 4 wk of therapy. If animals in any group became moribund, then the experiment was terminated and all animals were sacrificed together. Mouse weight, tumor weight, number of nodules, and tumor site were recorded. Tissue specimens were collected as described above.

Tissue staining for PCNA, microvessel density, and apoptosis. For immunohistochemical analysis, paraffin-embedded tissues were sectioned (8 μm thick) and used to detect expression of PCNA. Frozen sections were used for detecting CD31 and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL). Generally, formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and treated with a graded series of alcohol. Antigen retrieval was done by microwave heating for 5 min at 95°C in 0.1 mol/L citrate buffer (pH 6.0) followed by blocking of endogenous peroxide with 3% hydrogen peroxide in methanol for 5 min. Incubation with primary antibody (anti-PCNA, PC-10, mouse IgG) in blocking solution was done overnight at 4°C. After two PBS washes, the appropriate secondary antibody conjugated to HRP in blocking solution was added for 1 h at room temperature. HRP was detected with DAB substrate for 5 min, washed, and counterstained with Gill No. 3 hematoxylin (Sigma) for 15 s. To analyze microvessel density (MVD) in tumor tissue, immunohistochemistry for CD31 was done on freshly cut frozen tissue. These slides were fixed in cold acetone for 10 min and did not require antigen retrieval. The primary antibody used was mouse anti-CD31 (platelet/endothelial cell adhesion molecule-1, rat IgG; PharMingen).

Figure 1. A, Western blot showing basal expression of TG2 protein in 11 ovarian cancer cell lines and one nontransformed normal ovarian epithelial cell line (HIO-180). B, transglutaminase enzymatic activity according to TG2 expression levels. Mean enzymatic activity of TG2 was determined in the cell extract by studying Ca²⁺-dependent incorporation of [³H]putrescine into dimethylcasein, as described in Materials and methods. Bars, SD. C, representative immunohistochemical staining of clinical specimens for TG2 expression. Original magnification, ×200. D, Kaplan-Meier survival curve of patients with invasive ovarian cancer according to TG2 expression.
For examination of the level of apoptosis in tumor tissue, TUNEL staining was done. Briefly, freshly cut frozen tissue was fixed in 4% paraformaldehyde in PBS for 10 min followed by the addition of 0.2% Triton X-100 for 15 min at room temperature. A positive control slide was treated with DNase (1:50) dilution. Slides were incubated with terminal deoxynucleotidyl transferase enzyme (1 µL/slide) for 1 h at 37°C followed by counterstaining with Hoechst. Staining for PCNA, CD31, and TUNEL was conducted on tumors collected at the conclusion of 3 or 4 wk of therapy.

**Microscopic quantitative analyses of PCNA, MVD, and TUNEL.** To quantify MVD, five random 0.159-mm² fields at ×100 final magnification per one slide were examined for each tumor (one slide per mouse, five slides per each treatment group) and the number of microvessels per field was counted by two investigators in a blinded fashion. A single microvessel was defined as a discrete cluster or single cell stained positive for CD31 with the presence of a lumen. To quantify PCNA expression, the number of PCNA-positive cells and the total number of tumor cells were counted in five random 0.159-mm² fields at ×100 magnification followed by calculation of the positive cell percentages. For quantification of TUNEL-positive tumors, the number of TUNEL-positive and the total number of tumor cells were counted in random 0.011-mm² fields at ×400 magnification followed by calculation of the positive cell percentages. DAB-stained sections were examined on a Microphot-FX microscope (Nikon) equipped with a three-chip charge-coupled device color video camera (model DXC990, Sony). Immunofluorescence microscopy was done using a Microphot-FXA microscope (Nikon). Images were captured using a cooled charge-coupled device color video camera (model DXC990, Sony). Microscopic quantitative analyses of PCNA, MVD, and TUNEL were conducted on tumors collected at the conclusion of 3 or 4 wk of therapy.

**Statistics.** For in vivo experiments, differences in continuous variables (mean body weight, tumor weight, number of nodules, MVD, PCNA, and TUNEL) were analyzed using the Student t test for comparing two groups and by ANOVA for multiple group comparisons, with P < 0.05 considered statistically significant. For values that were not normally distributed, the Mann-Whitney rank sum test was used. The SPSS software was used for all statistical analyses.

**Results**

**TG2 expression in ovarian cell lines and clinical samples.** We first examined TG2 expression using Western blot in multiple ovarian cell lines (Fig. 1A). The nontransformed HIO-180 cells had absent TG2 expression by Western blot analysis. Moderate to high TG2 expression was noted in HeyA8, HeyA8-MDR, RMG2, and ES2 cells. All of the remaining cell lines had either low or absent TG2 expression. Consistent with the expression data, TG2 enzyme activity was absent or low in A2780-PAR, A2780-CP20, and SKOV3ip1 cells. Conversely, HeyA8 and HeyA8-MDR cells showed high TG2 enzyme activity (Fig. 1B).

Based on high TG2 expression in several ovarian cancer cell lines, we next examined TG2 expression in 93 epithelial ovarian cancers using immunohistochemistry. Representative examples of TG2 staining are shown in Fig. 1C and correlations with clinicopathologic variables are listed in Table 1. The demographic features of the patients with invasive ovarian cancers are listed in Supplementary Table S1. TG2 overexpression was associated with high tumor stage (69% of high-stage ovarian cancers overexpressed TG2 compared with 30% of low-stage cancers; P = 0.01). TG2 expression was not related to histologic subtype (serous versus other), grade (low versus high), presence of ascites, or level of cytoreduction (optimal versus suboptimal). In univariate analysis, TG2 overexpression was associated with significantly worse patient survival (median survival of 2.29 years for those with high TG2 expression versus not yet reached for individuals with low expression; P = 0.007; Fig. 1D). In multivariate analysis including age, stage, grade, level of cytoreduction, and TG2 expression, only stage (P < 0.002), level of cytoreduction (P = 0.001), and TG2 overexpression (P < 0.04) were independent predictors of poor survival (Supplementary Table S2).

**The roles of TG2 on adhesion, invasion, and survival function of cancer cells.** Based on the effect of TG2 overexpression on patient outcome, we next characterized its biological effects on cancer cells. TG2 was ectopically overexpressed in SKOV3ip1 cells, which have low baseline levels of TG2, using TG2-W, TG2-M, or empty vector. The overall expression of TG2 in SKOV3ip1 cells transfected with TG2-W increased by 3- to 4-fold over the basal levels (Supplementary Fig. S1). First, we tested whether TG2 expression in ovarian cancer cells can promote adhesion to matrix because TG2 can serve as a coreceptor for integrin-mediated binding of cells to fibronectin (14). Infection of cells with empty vector had no effect on cell adhesion, whereas SKOV3ip1 cells transfected with TG2-W showed 1.7-fold increased attachment and spreading when cultured on fibronectin-coated plates (P < 0.001; Fig. 2A). On BSA-coated plates, however, they were far less adherent and showed a rounded morphology (data not shown). We also examined the effects of TG2 on the in vitro invasive potential of the transfected SKOV3ip1 cells. TG2-W transfection was associated with a parallel increase in the invasive potential of SKOV3ip1 cells (P < 0.001; Fig. 2B). Next, we determined if the catalytic (transamidation) activity of TG2 is essential for inducing invasiveness in SKOV3ip1 cells. Infection of cells with mutant-TG2-adenoviral construct (TG2-M), which codes enzymatically inactive TG2 protein as a result of point mutation in the active-site cysteine residue (Cys277Ser), promoted a similar increase in invasiveness of SKOV3ip1 cells (Fig. 2B). These results suggest that TG2 expression plays an important role in promoting the invasive phenotype in ovarian cancer cells and that the transamidation activity of TG2 is not essential for conferring these functions. Infection of cells with empty vector, however, had no effect on the invasive potential.

The interaction of cancer cells with ECM is known to induce cell survival signaling pathways and confer chemoresistance (27).

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Table 1. Correlation of clinicopathologic variables with TG2 overexpression in patients with invasive ovarian cancer

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Therefore, we examined whether TG2-mediated attachment of cells could confer protection from drug-induced cytotoxic effects. We found that SKOV3ip1 cells with TG2-W were more resistant to paclitaxel-induced cell death than controls (Fig. 2C). Empty vector–transfected cells were similar to nontransfected controls in response to paclitaxel (data not shown). These results suggest that TG2 expression in ovarian cancer cells promotes cell-surface interaction with ECM and protects cells from apoptosis.

It has been suggested that TG2 expression can induce constitutive activation of FAK and its downstream phosphatidylinositol 3-kinase/Akt survival pathway, which is independent of its enzymatic activity (19). Therefore, we examined the effects of TG2-W overexpression in SKOV3ip1 cells for activation of this pathway. Western blot analysis for pAkt (Ser473) and total Akt (tAkt) in SKOV3ip1 cells transfected with TG2-W or empty vector.

Figure 2. A, TG2 was ectopically introduced into SKOV3ip1 cells by adenovirus containing TG2 gene. Effects of TG2 overexpression on adhesion were examined following transfection of wild-type TG2 gene (TG2-W) or empty vector (EV) into SKOV3ip1 cells. Control represents untransfected cells. Left, pictures obtained from fibronectin-coated plates. Right, mean absorbance. Bars, SD. B, effect of wild-type TG2 gene (TG2-W) versus mutant type TG2 gene (TG2-M) on SKOV3ip1 invasive potential was examined with a Matrigel transwell assay. Each experiment was done in triplicate and repeated at least twice. C, SKOV3ip1 cells infected with TG2-W or without any treatment were plated on 96-well plates. After attachment, the medium was exchanged with increased concentrations of paclitaxel (0–25 nmol/L). The number of viable cells remaining was determined after 72 h. Bars, SD. D, Western blot analysis for pAkt (Ser473) and total Akt (tAkt) in SKOV3ip1 cells transfected with TG2-W or empty vector.

Based on the effects of ectopic TG2 expression on SKOV3ip1 invasion, we next asked whether TG2 silencing would block these effects. For these experiments, we used a siRNA targeted to TG2. First, we performed in vitro TG2 knockdown experiments using HeyA8 cells with high TG2 expression. TG2 expression was decreased by 80% at 48 hours after TG2 siRNA transfection (Fig. 3A; Supplementary Fig. S2). HeyA8 cells were highly invasive without transfection; however, the inhibition of TG2 by siRNA significantly decreased the ability of cells to invade through the Matrigel-coated transwell inserts (P < 0.001; Fig. 3B), suggesting that TG2 expression promotes invasive functions in ovarian cancer cells. In addition, knockdown of TG2 with siRNA increased docetaxel-induced cell death when the cells were cultured on fibronectin-coated plates (Fig. 3C). These results suggest that the TG2-dependent interaction between ovarian cancer cells and fibronectin is critical for inducing cell growth and survival.
In vivo silencing by TG2-specific siRNA-DOPC. Based on the evidence that the overexpression of TG2 in ovarian cancer cells is closely related to many aggressive tumor features, we investigated its potential as a therapeutic target. Based on our prior experience with in vivo gene silencing, we tested two doses of TG2 siRNA-DOPC in vivo. Nude mice bearing HeyA8 tumors were injected i.p. twice, 48 hours apart, with a dose of TG2 siRNA-DOPC 3.5 µg/mouse (105 µg/kg) or 5.0 µg/mouse (150 µg/kg) and tumors were harvested 2, 4, and 6 days after injection. With the 5.0 µg dose, a 70% decrease in TG2 levels was observed, which persisted for at least 4 days (Fig. 3D). TG2 expression began to return toward basal levels by 6 days after treatment. Similar results were noted with immunohistochemistry (Fig. 3D). TG2 expression was not affected by nonspecific control siRNA. Therefore, we selected the 5.0 µg (150 µg/kg) dose given twice weekly of TG2 siRNA-DOPC as the dosing schedule for subsequent therapy experiments.

In vivo therapy experiments with liposomal siRNA targeting TG2. HeyA8, RMG2, and HeyA8-MDR ovarian cancer cells were selected for the therapy experiments because these lines have high levels of TG2 expression. Seven days following tumor cell injection into the peritoneal cavity of nude mice, therapy was started according to five treatment groups: empty liposome, nonspecific control siRNA-DOPC, control siRNA-DOPC in combination with docetaxel, TG2 siRNA-DOPC, and TG2 siRNA-DOPC plus docetaxel. The animals were sacrificed after 3 to 4 weeks of therapy and a necropsy was done. In the HeyA8 model, treatments with control siRNA-DOPC plus docetaxel and TG2 siRNA-DOPC alone were effective in reducing tumor weight (P = 0.016 and P = 0.011, respectively; Fig. 4A). However, the greatest efficacy was observed with TG2 siRNA-DOPC plus docetaxel (91% reduction in tumor weight; P = 0.001). The combination therapy of TG2 siRNA-DOPC with docetaxel was more effective than control siRNA-DOPC plus docetaxel (P = 0.006) or TG2 siRNA-DOPC alone (P = 0.03). Given the high prevalence of resistance to chemotherapy in patients with ovarian cancer, we also examined the effects of TG2 silencing using the HeyA8-MDR model. As expected, docetaxel had no effect on tumor growth (Fig. 4B). However, TG2 siRNA-DOPC plus docetaxel reduced tumor weight significantly (P = 0.032). In the RMG2 model, treatments with TG2 siRNA-DOPC alone and TG2 siRNA-DOPC plus docetaxel were effective in reducing tumor weight (P = 0.029 and P = 0.001, respectively; Fig. 4C). However, the greatest efficacy was observed with TG2 siRNA-DOPC plus docetaxel (86% reduction in tumor weight). The combination therapy of TG2 siRNA-DOPC with docetaxel was more effective than control siRNA-DOPC plus docetaxel (P = 0.001) or TG2 siRNA-DOPC alone (P = 0.008).

Figure 3. A, in vitro knockdown of TG2 in HeyA8 cells showed that maximum down-regulation was seen at 48 h. B, effect of TG2 gene silencing on in vitro HeyA8 invasive potential. Each experiment was done in triplicate and repeated at least twice. C, effect of TG2 silencing on HeyA8 cell viability was examined with various concentrations of docetaxel (0–50 nmol/L). The number of viable cells remaining were determined after 72 h. Bars, SD. D, TG2 expression was assessed in HeyA8 tumors growing in the peritoneal cavity of nude mice following two doses of i.p. TG2 siRNA-DOPC. Top, Western blot. Bottom, immunohistochemical peroxidase staining for TG2. Original magnification, ×100.
Data from other measured variables of these therapy experiments are shown in Supplementary Table S3. The incidence of tumor formation was not significantly different among the five groups in either cell line. However, the number of nodules formed was reduced by treatment with control siRNA-DOPC plus docetaxel, TG2 siRNA-DOPC alone, and TG2 siRNA-DOPC plus docetaxel in HeyA8 cell–injected mice ($P = 0.018$, $P = 0.013$, and $P = 0.016$, respectively) and RMG2 cell–injected mice ($P = 0.015$, $P = 0.016$, and $P = 0.002$, respectively). We also examined TG2 expression at the conclusion of the therapy experiments by immunohistochemical staining analysis. TG2 expression levels were not affected by control siRNA-DOPC, empty liposome, or control siRNA-DOPC plus docetaxel. However, in the TG2 siRNA-DOPC and TG2 siRNA-DOPC with docetaxel groups, there was sustained suppression of TG2 expression at the end of the therapy experiments (Fig. 5A). There were no behavioral changes, such as change in eating habits or mobility, in animals treated with liposomal siRNA preparations, and mouse weights were not significantly different among the five groups of animals, suggesting that eating and drinking habits were not affected.

**Effect of TG2 targeting on cell proliferation, angiogenesis, and apoptosis.** To determine potential mechanisms underlying the efficacy of anti-TG2–based therapy, we examined its effects on several biological end points, including proliferation (PCNA), angiogenesis (MVD), and apoptosis (TUNEL). First, we examined the effects of TG2-targeted therapy on tumor cell proliferation by PCNA staining. A significant reduction of PCNA expression was observed in the groups treated with control siRNA-DOPC plus docetaxel, TG2 siRNA-DOPC alone, and TG2 siRNA-DOPC plus docetaxel ($all P < 0.01$; Fig. 5B). Next, we analyzed MVD (Fig. 5C) in the harvested tumors by CD31 immunostaining. Compared with the empty liposome group, the mean MVD was significantly reduced in tumors treated with TG2 siRNA-DOPC alone and TG2 siRNA-DOPC plus docetaxel ($P = 0.039$ and $P = 0.004$, respectively).
The most significant reduction in MVD occurred in the combination therapy group. To examine whether the TG2 siRNA-DOPC–mediated in vivo effects on endothelial cells could be direct, we treated murine endothelial cells isolated from the ovary of ImmortoMice [H-2k(b)-tsA58; ref. 28] and murine cancer cell lines with TG2 siRNA-DOPC. Murine TG2 levels were not altered by the TG2 siRNA-DOPC used for the in vivo experiments (data not shown). Finally, we evaluated tumor cell apoptosis by TUNEL staining (Fig. 5D). Minimal tumor cell apoptosis was apparent in empty liposome, control siRNA-DOPC, or control siRNA-DOPC with docetaxel treatment groups; however, treatment with TG2 siRNA-DOPC alone and TG2 siRNA-DOPC plus docetaxel resulted in a significant increase in apoptosis ($P = 0.027$ and $P = 0.001$, respectively). Interestingly, the increase in apoptosis in the TG2 siRNA-DOPC plus docetaxel group was greater than in the TG2 siRNA-DOPC alone group ($P = 0.004$).

**Discussion**

In this study, we found that overexpression of TG2 in ovarian carcinoma was significantly associated with worse overall patient survival. Moreover, TG2 promoted several biological functions of cancer cells such as cell attachment, invasion, and chemotherapy resistance. Furthermore, our in vivo experiments indicate that treatment with TG2 siRNA-DOPC plus chemotherapy was highly effective in reducing ovarian cancer growth.

In epithelial ovarian cancer, the most frequent and earliest route of spread is by exfoliation of cells that implant along the surfaces of peritoneal cavity. Based on the adhesion assays, TG2 may play a role in the early steps of ovarian cancer metastasis. These findings are further supported by recent observations about diminished dissemination of tumors on the peritoneal surface and mesentery following TG2 knockdown in an i.p. ovarian xenograft model (29). This phenotype was associated with deficient $\beta_1$ integrin-fibronectin interaction, leading to weaker anchorage of cancer cells to the peritoneal matrix.

Drug resistance and metastasis are major impediments for the successful treatment of ovarian cancer. A common feature among drug-resistant and metastatic tumor cells is that they exhibit profound resistance to apoptosis (30). Intracellular TG2 is able to cross-link the inhibitory subunit $\alpha$ of nuclear factor (I$\kappa$B$\alpha$) and, thus, constitutively activate the transcription factor nuclear factor $\kappa$B, which, in turn, promotes expression of antiapoptotic proteins such as Bcl-xL and BFL1 (31, 32). Kim and colleagues (33) reported that increased expression of TG2 and subsequent activation of nuclear factor $\kappa$B may contribute to drug resistance in breast...
cancer cells independently of epidermal growth factor signaling. Consistent with these findings, in vivo TG2 silencing with siRNA enhanced tumor cell apoptosis in our therapy models.

Although TG2 silencing seems to have direct antitumor effects, there is growing evidence that the tumor microenvironment may also be affected. Recently, it was shown that a plasma transglutaminase, thrombin-activated FXIII (FXIIIA subunit), activates vascular endothelial growth factor (VEGF) receptor 2 by cross-linking it with the α5β1 integrin on the surface of endothelial cells, thereby stimulating angiogenesis (34). In the current study, we found that treatment with TG2 siRNA-DOPC plus docetaxel decreased the MVD in tumor tissue. Further work is needed to determine the effect of TG2 on tumor angiogenesis. Recently, one study showed that TG2 expression in pancreatic cancer cells induced constitutive activation of FAK and its downstream phosphatidylinositol 3-kinase/Akt survival pathway and it was independent of its enzymatic activity (19). Consistent with this, our in vitro experiments showed that overexpression of TG2 in SKOV3ip1 cells transfected with TG2-W induced increased phosphorylation of Akt. FAK is a nonreceptor kinase that is a critical mediator of signaling between cells and their ECM (35). FAK activation at focal adhesion sites leads to cytoskeletal reorganization, cellular adhesion, and survival, and it is known to play a role in cell migration and invasion (22). In our previous experiments, FAK silencing was associated with lower levels of VEGF and matrix metalloproteinase 9 and increased apoptosis of tumor-associated endothelial cells, suggesting an antivascular effect. Therefore, TG2 may affect tumor angiogenesis indirectly by reducing FAK activation.

In another aspect, TG2 also plays certain roles in host protection and physiology. Several lines of evidence suggest that tissue transglutaminase plays an important role in stabilizing the ECM by cross-linking its component proteins and rendering it resistant to mechanical and proteolytic degradation (5, 9) and contributes to fibroblast wound healing processes (36). Furthermore, it was reported that TG2-induced alterations in the ECM of host could effectively inhibit the process of metastasis (37). Although TG2 silencing was very effective in treating ovarian cancer in our orthotopic models, we cannot fully determine the significance of tumor versus host TG2. Future studies using TG2-null background mice will be helpful in clarifying the role of host versus tumor TG2 in the progression of ovarian cancer.

TG2 overexpression has been implicated in the pathogenesis of a number of medical diseases such as celiac sprue (38), neurodegenerative disorders (39), diabetes (40), liver cirrhosis and fibrosis (41), and renal scarring (42), as well as various cancers. Although the current study was focused on ovarian cancer, TG2 silencing with siRNA incorporated in neutral liposomes may be useful for the management of other benign and malignant diseases. Several kinds of TG2 inhibitors are being developed for disease control such as competitive amine inhibitors, reversible inhibitors, and irreversible inhibitors (43). Recently, some preclinical data with TG2 inhibitors have been reported. For example, treatment of glioblastoma cells in culture with the competitive TG2 inhibitor monodansylcadaverine or with the selective small-molecule irreversible TG2 inhibitor KCA075 or its analogue KCC009 showed an increased incidence of tumor cell apoptosis (44). In addition, KCC009 treatment in mice harboring orthotopic glioblastomas sensitized the tumors to N,N′-bis(2-chloroethyl)-N-nitrosourea chemotherapy, as measured by reduced bioluminescence, increased apoptosis, and prolonged survival. Our systemic siRNA approach offers an attractive alternative option for therapeutic targeting of TG2.

In summary, targeted therapy with TG2 siRNA-DOPC in combination with chemotherapy significantly reduces tumor growth in both chemotherapy-sensitive and chemotherapy-resistant models. This antitumor effect was closely related to reduced proliferation, decreased angiogenesis, and increased tumor cell apoptosis, in addition to decreased attachment and invasion. Given the clinical relationship between TG2 and ovarian cancer prognosis, our findings raise the possibility that TG2 silencing in combination with docetaxel chemotherapy could be a novel therapeutic option against advanced ovarian carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

4. Mehta K. Transglutaminase silencing was very effective in treating ovarian cancer in our in vivo experiments showed that overexpression of TG2 in SKOV3ip1 cells transfected with TG2-W induced increased phosphorylation of Akt. FAK is a nonreceptor kinase that is a critical mediator of signaling between cells and their ECM (35). FAK activation at focal adhesion sites leads to cytoskeletal reorganization, cellular adhesion, and survival, and it is known to play a role in cell migration and invasion (22). In our previous experiments, FAK silencing was associated with lower levels of VEGF and matrix metalloproteinase 9 and increased apoptosis of tumor-associated endothelial cells, suggesting an antivascular effect. Therefore, TG2 may affect tumor angiogenesis indirectly by reducing FAK activation.

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34. Dardik R, Inbal A. Complex formation between tissue transglutaminase II (TG2) and vascular endothelial growth factor receptor 2 (VEGFR-2): proposed mechanism for modulation of endothelial cell response to VEGF. Exp Cell Res 2006;322:297–83.


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