Implications of tissue transglutaminase expression in malignant melanoma

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Abstract
Human malignant melanoma is a highly aggressive form of cancer; the 5-year survival rate in patients with stage III or IV disease is <5%. In patients with metastatic melanoma, systemic therapy becomes ineffective because of the high resistance of melanoma cells to various anticancer therapies. We have found previously that development of the drug resistance and metastatic phenotypes in breast cancer cells is associated with increased tissue transglutaminase (TG2) expression. In the study reported here, we investigated TG2 expression and its implications in metastatic melanoma. We found that metastatic melanoma cell lines expressed levels of TG2 up to 24-fold higher than levels in radial growth phase of primary melanoma cell lines. Activation of endogenous TG2 by the calcium ionophore A23187 induced a rapid and strong apoptotic response in A375 cells and A23187-induced apoptosis could be blocked by TG2-specific inhibitors. These findings indicated that activation of endogenous TG2 could serve as a strategy for inducing apoptosis in malignant melanomas. Importantly, tumor samples from patients with malignant melanomas showed strong expression of TG2, suggesting that TG2 expression is selectively up-regulated during advanced developmental stages of melanoma. We observed that 20% to 30% of TG2 protein was present on cell membranes in association with β1 and β5 integrins. This association of TG2 with cell surface integrins promoted strong attachment of A375 cells to fibronectin-coated surfaces, resulting in increased cell survival in serum-free medium. Inhibition of TG2 by small interfering RNA inhibited fibronectin-mediated cell attachment and cell survival functions in A375 cells. Overall, our results suggest that TG2 expression contributes to the development of chemoresistance in malignant melanoma cells by exploiting integrin-mediated cell survival signaling pathways. [Mol Cancer Ther 2006;5(6):1493–503]

Introduction
Melanoma is the deadliest form of skin cancer and its incidence is rapidly growing (1). In stage I and II disease, complete surgical excision of the primary tumor is associated with a success rate of >95%. However, in stage III (lymph node infiltration) and stage IV (distant metastasis) disease when melanoma has disseminated to multiple organ sites, including brain, lungs, liver, and bone, surgical interventions are of limited use. Moreover, systemic therapy has minimal success because of the high intrinsic resistance exhibited by melanoma cells against various anticancer therapies.

A large number of genetic, functional, and biochemical studies suggest that melanoma cells become resistant to chemotherapy by exploiting their intrinsic resistance to apoptosis (2). Alterations that contribute to the development of resistance to apoptosis can enable cancer cells not only to survive under stressful conditions (e.g., during metastasis) but also to develop resistance to drugs (3, 4). Therefore, elucidation of novel proteins and pathways that contribute to the development of resistance to apoptosis may reveal promising molecular targets for effective treatment of melanomas.

We have found previously that development of the drug resistance phenotype in several types of cancer cells is associated with increased expression of tissue transglutaminase (TG2; refs. 5–9). TG2 (EC2.3.2.13), also called the cytosolic, type II, or liver transglutaminase, is a unique member of the transglutaminase enzyme family. In addition to catalyzing the calcium-dependent protein cross-linking reactions (10–12), TG2 can catalyze calcium-independent hydrolysis of GTP and ATP (10–12), protein disulfide isomerase reactions (13, 14), and serine/threonine kinase activity (15–17). Although, it is predominantly a cytosolic protein, TG2 also can be secreted outside the cell (17, 18), can translocate to the nucleus with the help of importin-α3 protein (19), and can be expressed on the cell membrane in association with β members of the integrin family of proteins (20–22). Cell surface TG2 promotes adhesion and spreading of cells, enhances focal adhesions, and amplifies adhesion-dependent phosphorylation of focal adhesion kinase (5, 20, 21).

The integrins, in association with which TG2 can be expressed on the cell surface, can strongly influence the ability of neoplastic cells to migrate, proliferate, undergo apoptosis, and mediate invasion and metastasis (20–26). Integrins differ from other cell surface receptors in that
they bind their ligands in the extracellular matrix with a low affinity. However, in response to certain stimuli, integrins can cluster (in focal contacts), and their combined weak affinities then give rise to a spot on the cell surface that has enough adhesive capacity (avidity) to promote stable attachment to the extracellular matrix. Alternatively, certain proteins can bind directly to integrins and enhance their affinity for the extracellular matrix ligands, thereby promoting cell signaling (27, 28).

In the present study, we investigated TG2 expression and its implications in metastatic melanoma. We showed that malignant human melanoma cells and cell lines express high basal levels of TG2. Activation of endogenous TG2 by the calcium ionophore A23187 led to a massive cross-linking of cellular proteins and spontaneous apoptosis in these cells. TG2 was also localized on the cell surface of malignant melanoma cells in association with $\beta_1$ and $\beta_5$ integrins. Cell surface TG2 played an important role in promoting attachment and protecting cells from apoptosis when cells were cultured on fibronectin or its 110-kDa fragment. Taken together, these results suggest that activation of constitutively expressed TG2 is an effective strategy for selectively inducing apoptosis in malignant melanoma cells and that TG2 expression contribute to the development of chemoresistance in malignant melanoma cells by exploiting integrin-mediated cell survival signaling pathways.

Materials and Methods

**Cell Lines and Patient Samples**

Melanoma tissue samples from patients with various stages of disease were surgically removed from patients enrolled in institutionally approved trials. Formalin-fixed, paraffin-embedded tissue sections were obtained from the Melanoma and Skin Cancer Core Laboratory of The University of Texas M.D. Anderson Cancer Center (Houston, TX) for use in immunohistochemical labeling of tumor tissues.

Metastatic melanoma cell lines, A375 and A375-S2, were obtained from American Type Culture Collection (Manassas, VA). Primary melanoma cell lines, WM35 (radial growth phase) and WM793 (vertical growth phase), were kindly provided by Dr. Robert Kerbel (Sunnybrook Health Science Center, Toronto, Ontario, Canada), and MeWo (metastatic) cell line was provided by Dr. Elizabeth A. Grimm (The University of Texas M. D. Anderson Cancer Center). All the cell lines were maintained in a log phase of cell growth by culturing in RPMI 1640 supplemented with 10% FCS, 0.2% normocin (Invivogen, San Diego, CA), 2 mmol/L L-glutamine, and 10 mmol/L HEPES at 37°C in the CO2 incubator. Normal human epidermal melanocytes were obtained from Clonetics Corp. (San Diego, CA) and were cells were maintained according to the manufacturer’s instructions. Samples of formalin-fixed, paraffin-embedded human benign nevi were retrieved from the Department of Surgical Pathology.

**Measurement of TG2 Enzyme Activity in Cell Lysates**

Cells grown to 90% confluence were collected in a minimal volume (100-300 µL) of buffer A (20 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, 150 mmol/L NaCl, 14 mmol/L 2-mercaptoethanol, and a 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)) and lysed in the same buffer by probe sonication (8-10 pulses of 10 seconds, each). Protein contents of the cell lysates were determined using dye reagent (Bio-Rad, Richmond, CA). Cell lysates were assayed for TG2 activity by determining the Ca2+-dependent incorporation of [3H]putrescine (specific activity, 14.3 Ci/mmol; Amersham Pharmacia, San Francisco, CA) into dimethylcasein as described previously (6). The enzyme activity was expressed as nanomoles of putrescine incorporated per hour per milligram of total lystate protein.

**Western Blotting**

Cell lystate protein (30 µg) was separated by SDS-PAGE on a 7.5% gel and electrophoretically transferred onto a nitrocellulose membrane. The membrane was probed with anti-TG2 monoclonal antibody (CUB7401; NeoMarkers, Fremont, CA) or anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody reaction was detected using an enhanced chemiluminescence detection system (Pierce, Rockford, IL). All the membranes were stripped and reprobed with an anti-$\beta$-actin antibody (Sigma-Aldrich) at a dilution of 1:4,000 to ensure even loading of proteins in different lanes.

**Measurement of TG2 Activity in Intact Cells**

TG2 activity in intact cells was determined by preincubating cells with 1 mmol/L 5-(biotinamido) pentylamine (BPA), a competitive inhibitor of TG2-catalyzed cross-linking reactions, in 2% FCS overnight at 37°C. To induce activation of endogenous TG2, cells were treated with the calcium ionophore A23187 (4 µmol/L, 4 hours). Cells were then washed and lysed by sonication in 500 µL buffer A. Equal amounts of cell lysate proteins were fractionated by SDS-PAGE on an 8% gel and electrophoretically transferred onto a nitrocellulose membrane. The membrane was probed with horseradish peroxidase (HRP)–conjugated streptavidin (Sigma-Aldrich) and then with enhanced chemiluminescence reagent.

Similarly, the ability of endogenous cellular proteins to serve as substrates for endogenous TG2-catalyzed cross-linking reactions was tested as described previously (29). Briefly, cell extracts containing equal amounts (30 µg) of proteins were incubated in a total volume of 200 µL buffer A containing 1 mmol/L BPA and 3 mmol/L of either CaCl2 or EDTA (background control). The reaction mixture (30 µL) was removed at different time points and immediately mixed with 3× sample buffer to stop the reaction. Reaction mixtures (equivalent to 30 µg cell protein) were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was probed with HRP-conjugated streptavidin and then with enhanced chemiluminescence reagent.

**Measurement of Cell Growth and Attachment**

Ninety-six-well plates (Corning/Costar, Rochester, NY) were coated with 20 µg/mL fibronectin (Sigma-Aldrich) or its 110- or 42-kDa fragment (both fragments were kindly provided by Dr. Alexey Belkin, University of Maryland,
Baltimore, MD) or 0.1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS. The nonspecific binding sites were blocked with 2% BSA. The cells were grown in T-75 flasks to 80% to 90% confluence isolated by trypsinization, washed once with RPMI 1640, and resuspended at 5 × 10^5 cells/mL in serum-free RPMI 1640. Aliquots (200 μL) of the cell suspension were added to fibronectin- or BSA-coated wells in quadruplicate and incubated at 37°C for 48 hours. At the end of the incubation period, the number of viable cells remaining in the well was determined by measuring their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium into soluble formazan.

In some experiments, A375 cells were transfected with TG2-specific [small interfering RNA (siRNA) 1 and 2] or control (scrambled) siRNAs using RNAiFect reagent (Qiagen Sciences, Germantown, MD) in accordance with the manufacturer’s instructions. After 48 hours of transfection, the cells were harvested with 2 mmol/L EDTA, washed, and preclarified lysates were incubated overnight at 4°C. Beads were washed with the extraction buffer and eluted with 2× SDS-PAGE sample buffer. The eluted proteins were analyzed with Western blotting. To detect TG2 in the immunoprecipitates, membranes were first probed with anti-TG2 (M-300; Neomarkers) antibody and then with anti-rabbit IgG-HRP. The membrane was then stripped and reprobed with anti-β1 or anti-β3 integrin antibodies.

**Measurement of Apoptosis**

Cells (1 × 10^6) were seeded into 25-cm² tissue culture flasks. Two days later, the cultures were incubated with RPMI 1640 (containing 2% FCS) alone or medium containing A23187. At predetermined time points, cells were viewed under phase-contrast microscope and photographed. In some experiments, total (floating and adherent) cell populations were collected and centrifuged (800 × g, 5 minutes), and the pellets were immediately processed for further experimentation. To determine the effect of TG2 inhibition on A23187-induced apoptosis, cells were treated with 1 mmol/L BPA overnight before treatment with A23187.

In some experiments, apoptosis was also determined by using the ApoAlert Annexin kit (BD Biosciences, San Diego, CA). Briefly, after appropriate treatment, cells were washed in PBS and suspended (1 × 10^6/mL) in binding buffer. For each 150 μL cell suspension, 5 μL Annexin V Cy5 and 10 μL propidium iodide were added and incubated for 15 minutes in the dark at room temperature. Ten thousand events were counted by flow cytometry.

**Flow Cytometry**

Cells were detached with 2 mmol/L EDTA, washed, and resuspended (2 × 10^6/mL) in PBS containing 0.1% BSA. Cell suspensions (0.1 mL) were incubated with primary antibodies specific to various integrins (anti-integrin β1, MAB 1987Z; β3, MAB 1957; β5, MAB 1926; αv, MAB 1980; or α6, MAB 1956Z; all from Chemicon, Temecula, CA) or TG2 (anti-TG2 monoclonal antibody CUB7401) on ice for 30 minutes and then washed twice with ice-cold PBS. The fluorochrome-labeled secondary antibodies (goat anti-mouse immunoglobulin G Alexa 546 or goat anti-rabbit IgG Alexa 488; both from Molecular Probes, Eugene, OR), which indicated antigen-antibody reaction, were detected using a FACScan flow cytometer. For the control setting for each cell type, isotypic IgG along with secondary antibody was used.

**Immunoprecipitation**

Cells were lysed in extraction buffer containing 20 mmol/L Tris-HCl (pH 7.6), 100 mmol/L NaCl, 0.5% NP40, and 5 mmol/L EDTA supplemented with protease inhibitors. Total cell lysate protein (400 μg) was preclarified by incubation with protein G-Sepharose beads. The preclarified lysates were incubated overnight at 4°C with anti-β1 or anti-β3 integrin antibody (Chemicon). The next day, antigen-antibody complexes were removed by incubating the solutions with either anti-mouse IgG or anti-rabbit IgG (1 hour at 4°C) and then protein G-Sepharose beads (1-3 hours at 4°C). Beads were washed with the extraction buffer and eluted with 2× sample buffer. Bound proteins were analyzed with Western blotting. To detect TG2 in the immunoprecipitates, membranes were first probed with anti-TG2 (M-300; Neomarkers) antibody and then with anti-mouse IgG-HRP. The membrane was then stripped and reprobed with anti-β1 or anti-β3 integrin antibodies.

**Immunofluorescence Staining**

Cells (2 × 10^6) were cultured on glass coverslips in six-well plates, rinsed thrice with PBS, fixed with 3.7% paraformaldehyde for 1 hour, and blocked with 5% normal goat serum for 1 hour. The cells were immunostained by using primary antibodies specific to various integrins and TG2. Goat anti-mouse IgG Alexa 488 or Alexa 546 or goat anti-rabbit IgG Alexa 488 or Alexa 546 was used as the secondary antibody. The stained coverslips were mounted on glass microscope slides in mounting medium (80% glycerol plus 20% PBS). Images were taken under a Nikon Eclipse fluorescence microscope (Melville, NY) using MetaFluor software (Universal Imaging Corp., Downingtown, PA).

Similarly, paraffin-embedded tissue sections from selected tumor samples were deparaffinized and immunostained with primary rabbit anti-TG2 and mouse anti-β1 or anti-β3 integrin antibodies. Goat anti-mouse IgG Alexa 488 and goat anti-rabbit IgG Alexa 488 or Alexa 546 were used as the secondary antibodies. Stained sections were mounted with mounting medium and viewed under a light microscope (Nikon). Appropriate controls (mouse and rabbit IgG in place of the primary antibodies and either primary antibody alone along with both of the secondary antibodies) were included to determine the specificity of the reaction.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tumor sections (5 μm thick) were immunostained for TG2. Briefly, after antigen
retrieval, tissue sections were incubated with anti-TG2 monoclonal antibodies overnight at 4°C and then incubated for 30 minutes each with biotinylated secondary antibody and peroxidase-labeled streptavidin. Antigen-antibody reactions were detected by exposure to 3,3’-diaminobenzidine and hydrogen peroxide chromogen substrate (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin and mounted. The negative controls were incubated with nonimmune mouse IgG in place of the primary antibody.

**Results**

**TG2 Expression in Normal Melanocytes and Melanoma Cell Lines**

We first determined the TG2 expression in epidermal melanocytes isolated from normal skin and in five malignant human melanoma cell lines representing various stages of disease progression: WM793 (vertical growth phase), WM35 (early stage), MeWo (metastatic), A375-S2 (metastatic), and A375 (highly metastatic amelanotic). The melanocytes isolated from normal human skin showed complete lack of TG2 as determined by Western blotting (Fig. 1A, lane 6) and enzymatic activity (Fig. 1B). Similarly, WM35 cells representing early-stage disease (radial growth phase) showed little TG2 expression (Fig. 1A and C) or enzyme activity (2.6 ± 0.4 nmol/h/mg). A375 cells, on the other hand, representing highly metastatic amelanotic malignant melanoma, showed a 20- to 25-fold increase in TG2 expression and enzyme activity compared with normal epidermal melanocytes and WM35 cells (Fig. 1). The other cell lines representing advanced-stage and metastatic melanoma also had high TG2 expression and enzyme activity.

**TG2 Expression and Sensitivity to Chemotherapy in Melanoma Cell Lines**

Next, we determined whether the level of expression of TG2 in melanoma cell lines corresponded with their sensitivity to the cytotoxic effects of cisplatin and dacarbazine. We tested the effect of cisplatin and dacarbazine on the viability of the five cell lines (Fig. 2). In quadruplicate, wells in 96-well plates containing 2,000 cells per well in 0.2 mL RPMI 1640 were either left untreated or treated with the indicated concentrations of cisplatin (A) or dacarbazine (B). Forty-eight hours after treatment, viable cells remaining in wells were determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium assay, and the percentage viability was calculated. Experiments were repeated at least three times with similar results. Points, mean of quadruplicate values from a representative experiment; bars, SD.

**Figure 1.** TG2 expression and enzyme activity in normal melanocytes and melanoma cell lines. A, TG2 expression was determined by Western blotting using the anti-TG2 monoclonal antibody CUB7401 as a probe. The nitrocellulose membrane was stripped and reprobed with anti-β-actin antibody to ensure even loading of lanes. Cell extracts (30 μg each) were loaded as follows: lane 1, A375; lane 2, A375-S2; lane 3, MeWo; lane 4, WM35; lane 5, WM793; lane 6, normal melanocytes. B, TG2 enzyme activity was determined in the cell extracts by studying Ca2+-dependent incorporation of [3H]putrescine into dimethylcasein as described in Materials and Methods. Columns, mean of six values from two independent experiments; bars, SD. C, immunofluorescence microscopy images of WM35 and A375 cells immunostained with TG2-specific anti IgG1.

**Figure 2.** Sensitivity of melanoma cells to cisplatin and dacarbazine. In quadruplicate, wells in 96-well plates containing 2,000 cells per well in 0.2 mL RPMI 1640 were either left untreated or treated with the indicated concentrations of cisplatin (A) or dacarbazine (B). Forty-eight hours after treatment, viable cells remaining in wells were determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium assay, and the percentage viability was calculated. Experiments were repeated at least three times with similar results. Points, mean of quadruplicate values from a representative experiment; bars, SD.
Activation of TG2 as a Target for Inducing Apoptosis in Melanoma Cells

Because it is well known that activation of endogenous TG2 can induce apoptosis in various cell types (10–12, 30), we next determined whether activation of endogenous TG2 could serve as a strategy for killing malignant melanoma cells. Treatment with calcium ionophore A23187, which activates TG2, induced significant cell killing in all the cell lines as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium assay after 48 h of treatment. Points, mean of quadruplicate values from a representative experiment; bars, SD. Experiments were repeated at least twice with similar results. B, photomicrographs showing morphologic changes induced in response to treatment of WM35 and A375 melanoma cells for 24 and 48 h with A23187 (2 μmol/L).

Figure 3. Effect of treatment with the calcium ionophore A23187 on malignant melanoma cells. A, dose-dependent cytotoxicity of A23187 against malignant melanoma cells as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium assay after 48 h of treatment. Points, mean of quadruplicate values from a representative experiment; bars, SD. Experiments were repeated at least twice with similar results. B, photomicrographs showing morphologic changes induced in response to treatment of WM35 and A375 melanoma cells for 24 and 48 h with A23187 (2 μmol/L).

Figure 4. Calcium ionophore A23187-induced apoptosis in A375 and WM35 melanoma cells. A, cells grown to 80% confluence were incubated in either medium alone or medium containing 4 μmol/L A23187. Twenty h later, cells were harvested and stained for Annexin V and analyzed by flow cytometry to determine the number of apoptotic cells as described in Materials and Methods. B, cells were left untreated or treated with 4 or 8 μmol/L A23187 for 20 h. After the treatment, cells were harvested and analyzed for Annexin V staining by flow cytometry. Representative experiment repeated twice with <20% SD. C, after 24- and 48-h treatment with 2 μmol/L A23187, cells were harvested and the cell lysates (30 μg/lane) were analyzed for caspase-3 activation by Western blotting. Cells incubated in medium alone for 48 h (-) served as control. The nitrocellulose membrane was treated with strip buffer and reprobed with anti-β-actin antibody to ensure even loading of proteins in different lanes.
Significantly more susceptible to A23187-induced apoptosis than were TG2-low WM35 cells (Fig. 4A and B). The Annexin V labeling data also correlated with caspase-3 activation; under identical conditions, caspase-3 activation was more rapid and more pronounced in A375 cells than in WM35 cells (Fig. 4C).

Next, we determined whether A23187-induced apoptosis in A375 cells was related to activation of endogenous TG2. To test this, we first looked for cellular proteins in A375 and WM35 cell lysates that could serve as substrates for endogenous TG2. Equal amounts of cell proteins were incubated in the presence of BPA (a competitive inhibitor of TG2-catalyzed protein cross-linking reactions) in the presence of either 5 mmol/L Ca\textsuperscript{2+} or EDTA (background control). Reaction mixtures were subjected to immunoblotting and the membranes were probed with HRP-streptavidin as described in Materials and Methods. The results showed numerous proteins that in a calcium-dependent manner could serve as substrates for endogenous TG2 in both cell types (Fig. 5A). MCF-7 cells that lack TG2 expression (6) did not show any BPA labeling in presence or absence of calcium (Fig. 5A, lanes 1 and 4). More importantly, treatment of A375 and WM35 cells with A23187 in the presence of BPA resulted in significant labeling of cellular proteins (Fig. 5B). Cells treated with BPA alone in the absence of A23187 failed to show any labeling of the cellular proteins, suggesting that the labeling of cellular proteins is mediated by activated TG2 in response to increased calcium levels induced by A23187 treatment. In general, A23187-induced incorporation of BPA was much more pronounced in TG2-rich A375 cells than in TG2-low WM35 cells (Fig. 5B).

To confirm direct involvement of endogenous TG2 in A23187-mediated killing of A375 cells, we next studied the effect of BPA on A23187-induced apoptosis. A375 and WM35 cells were incubated with 4 μmol/L A23187 in the presence or absence of 1 mmol/L BPA for 20 hours and analyzed for accumulation in sub-G1 phase of cell cycle (apoptotic fraction) using flow cytometry. Treatment with A23187 resulted in ~32% apoptosis in A375 cells (Fig. 5C). However, the presence of BPA during A23187 treatment significantly attenuated the extent of apoptosis in these cells (P < 0.001). These results suggested that TG2-catalyzed protein cross-linking reactions play an essential role in execution of A23187-induced apoptosis.

Interestingly, in TG2-low WM35 cells, the extent of A23187-induced apoptosis was significantly less than in A375 cells and BPA failed to rescue these cells from apoptosis (Fig. 5C).

**TG2 Expression in Tumor Samples from Patients with Melanoma**

Next, we determined the status of TG2 expression in tumor samples from a small cohort of patients (n = 12) with various stages of tumor progression. In normal skin, TG2 expression was restricted to the basal layer only (Fig. 6A), whereas both primary (Fig. 6B) and metastatic (Fig. 6C) melanomas showed high levels of TG2 expression.
Association of TG2 with Cell Surface Integrins

Because it has been shown previously that TG2 can be expressed on the cell membrane in association with β members of the integrin family of proteins (5, 20–22), we wished to determine whether that was the case for TG2 in melanoma cells. We first compared the cell surface expression of various integrins in WM35 and A375 cells. The surface expression of β1 integrin on TG2-rich A375 and TG2-low WM35 cells was similar (Fig. 7). However, the expression of other integrins was considerably higher (2- to 5-fold) in A375 cells than in WM35 cells (Fig. 7). In addition, the surface expression of TG2 was much stronger in A375 cells than in WM35 cells (Fig. 7).

Based on these results and the results published earlier (5, 20, 21), we hypothesized that TG2 may closely associate with integrins in A375 cells. To test this contention, immunoprecipitates from A375 and WM35 cells were isolated using anti-β1 or β5 integrin antibody and tested for the presence of TG2 protein. The results clearly established that anti-β1 and anti-β5 integrin antibody effectively pulled down TG2 protein in addition to integrins (Fig. 8A and B). These results suggested that TG2 protein is closely associated with β integrins on cell surface membranes of A375 cells.

Association of TG2 with β1 and β5 integrins was further supported by colocalization studies using fluorescence microscopy. As shown in Fig. 8C, TG2 (green fluorescence) colocalized with β1 and β5 integrins (red fluorescence) in A375 cells as evidenced by the yellow fluorescence in the merged images. Similarly, TG2 colocalized with these integrins in a limited number of patient samples (n = 3) that were tested for this purpose (Fig. 8D). Previously, we observed a similar association of TG2 with β1 and β5 integrins in drug-resistant MCF-7 breast cancer cells (5).

Because TG2 has high binding affinity for the 42-kDa gelatin-binding domain of fibronectin (31, 32) and is closely associated with integrins on the cell surface, we next examined whether TG2 expression could promote fibronectin-mediated cell attachment and signaling in malignant melanomas. Indeed, incubation of TG2-positive A375 cells on fibronectin-coated surfaces resulted in stronger adherence than did incubation of A375 cells on BSA-coated surfaces (Fig. 9A). WM35 cells, which express low levels of TG2 protein, showed weak attachment to both fibronectin- and BSA-coated surfaces (data not shown). The 110-kDa fragment of fibronectin promoted strong adherence of A375 cells (Fig. 9A) but not of WM35 cells (data not shown). The 42-kDa fragment of fibronectin, however, failed to support the attachment of either A375 or WM35 cells; adherence of cells on surfaces coated with 42-kDa fragment was similar...
to that on BSA-coated control (Fig. 9A; data not shown). Two antibodies against TG2 (CUB7401 and rabbit polyclonal antibody) did not affect the attachment of A375 or WM35 cells on surfaces coated with fibronectin or the 110- or 42-kDa fragments thereof (data not shown). In contrast, the function blocking anti-β1 integrin (JB1A) strongly blocked the adhesion of A375 cells to fibronectin and its 110-kDa fragment (data not shown).

To further analyze the involvement of TG2 in fibronectin-mediated cell attachment, we used a siRNA-based approach (5). We first tested the ability of siRNAs to down-regulate TG2 protein in A375 cells. The transfection efficiency of siRNAs as determined by fluorescein-labeled nonspecific siRNA was consistently 70% to 90%. The transfection with control siRNA (scrambled) had no appreciable effect on TG2 level (Fig. 9B). However, specific knockdown of TG2 with individual siRNAs (siRNA1 and siRNA2) suppressed the protein level by >70% (Fig. 9B). We then analyzed the effect of TG2 knockdown on fibronectin-mediated cell attachment. Inhibition of TG2 by siRNA effectively blocked the fibronectin-mediated attachment and spreading of A375 cells (Fig. 9C and D). No such effects were observed when the cells were transfected with control nonspecific (scrambled) siRNA. These results suggested that TG2 plays an important role in promoting the attachment and spreading of A375 melanoma cells to fibronectin-coated surfaces.

In a parallel experiment, untreated and siRNA-transfected A375 cells were continued for an additional 48 hours in serum-free medium on fibronectin- and BSA-coated plates. After 48 hours, the ability of TG2 and fibronectin to support cell growth and cell survival in the presence of serum-free medium was tested by determining the number of viable cells remaining in wells by crystal violet staining. TG2-rich A375 cells cultured on fibronectin-coated surfaces could survive and grow effectively under serum-free conditions (Fig. 10). However, on BSA-coated surfaces, these cells failed to survive (data not shown). More importantly, knockdown of TG2 in A375 cells with siRNAs markedly reduced the survival and growth of these cells even on fibronectin-coated surfaces (Fig. 10). These results suggested that TG2-dependent interaction between malignant melanoma cells and fibronectin is critical for inducing cell survival and cell growth signaling.

Discussion

This study shows that expression of TG2 is up-regulated during advanced stages of malignant melanomas. TG2 expression promotes cell attachment and integrin-mediated cell survival signaling on fibronectin-coated surfaces. Importantly, activation of endogenous TG2 results in rapid apoptosis, indicating that activation of endogenous TG2 could serve as a strategy for killing malignant melanomas.

Depending on the cell type and the location of TG2 within the cell, TG2 can serve as a proapoptotic or an antiapoptotic protein (7, 33–36). Although predominantly a cytosolic protein, TG2 can also localize in the nucleus (19) where it seems to associate with a variety of proteins, such as pRb, p53, and histones, and can regulate cellular functions (15–17). In association with the β subunits of...
the integrin family of proteins, TG2 can localize to the cell membrane and facilitate adhesion, spreading, and motility of cells (22, 37, 38). It is estimated that all TG2 on the cell surface is present in a 1:1 complex with integrins (20, 21).

We recently reported that drug-resistant and metastatic breast cancer cells exhibit high levels of TG2 (5–9, 39). Although the general consensus is that drug resistance and metastasis represent different phenotypes, it is well known that increased resistance to apoptosis is an important feature of both phenotypes (2–4). In view of this, it is tempting to speculate that high basal expression of TG2 in malignant melanomas promotes integrin-mediated signaling that affects not only the cell-adhesive, migratory, and invasive functions of these cells but also their survival and growth. Indeed, in the study reported here, we found that presence of TG2 promoted strong attachment of melanoma cells to fibronectin and its 110-kDa fragment. The attachment of cells could be effectively blocked by knocking down TG2 expression using a siRNA approach (Fig. 9). These results suggest that TG2 expression promotes stable interaction between integrins and fibronectin, the major protein in the extracellular matrix that plays an important function in inducing cell growth and cell survival signaling (40). Several previous studies have underscored this critical role for fibronectin. For example, culture of α5β1 integrin-expressing cells on fibronectin is associated with increased expression of the anti-apoptotic protein Bcl-2 and protection of cells from apoptosis in response to various stresses (41). Similarly, several cancer cell lines have been shown to be more resistant to chemotherapy- and radiation-induced cell death when they are cultured on fibronectin-coated surfaces (42–44).

Based on these observations and the observation that TG2 is closely associated with integrins β1 and β5 in melanoma cells (Fig. 8), it is tempting to speculate that TG2 expression contributes to chemoresistance and radiation resistance in malignant melanomas. Indeed, we found that knockdown of TG2 by siRNA in A375 cells strongly influenced their ability to attach to the fibronectin-coated surfaces and to survive under serum-free conditions (Fig. 10). In recent years, evidence supporting a role of TG2 in protecting cells from apoptosis has been emerging (7, 33–36). For example, treatment of breast cancer cells with epidermal growth factor was shown to induce TG2 expression that in turn rendered the cells resistant to chemotherapeutic drugs (45). Previous studies by our group have shown that irrespective of the type and source of cells development of the drug resistance phenotype in cancer cells is associated with

**Figure 9.** Attachment and spreading of A375 cells on fibronectin-coated surfaces is mediated by TG2. **A**, A375 cells were seeded in 96-well plates coated with BSA, fibronectin (Fn), or 110- or 42-kDa fragments of fibronectin (2 × 10⁴ per well; 0.2 mL serum-free medium). After 1-h incubation, cells were analyzed for attachment after washing and staining with crystal violet as described in Materials and Methods. **Columns**, averages of six values from two independent experiments; **bars**, SD. **B**, A375 cells were transfected with TG2-specific siRNAs (siRNA1 and siRNA2) or control (scrambled) siRNA. After 48 h, cells were harvested and analyzed for TG2 enzyme activity and TG2 protein levels (inset; lane 1, untreated; lane 2, control siRNA; lane 3, siRNA1; lane 4, siRNA2). Untreated WM35 cells were used as control. **C**, untreated (1) and siRNA-transfected (2, control siRNA; 3, siRNA1; 4, siRNA2) A375 cells were incubated in quadruplicate on fibronectin- or BSA-coated plates. After 1-h incubation, nonadherent cells were removed and adherent cells were examined under light microscope or stained with crystal violet (D) for quantitative analysis.
likely that under extremely stressful conditions massive irreversible cross-link proteins in the presence of Ca\textsuperscript{2+}. It is functions of TG2 are linked to the ability of TG2 to have a proapoptotic role (12–15). The proapoptotic and conferring chemoresistance, TG2 has also been shown to induce strong activation of the focal adhesion kinase on fibronectin-coated surfaces has been shown previously that knocking down TG2 protein by increased TG2 expression (5–9, 39). Importantly, we found in an earlier study that inhibition of TG2 by TG2-specific siRNA restored sensitivity to doxorubicin in drug-resistant MCF-7 breast cancer cells (5). A similar reversal in sensitivity to doxorubicin was noted by Han and Park (46) in drug-resistant PC-14 lung cancer cells in response to TG2 inhibition by an antisense approach. These observations strongly suggest that TG2 expression can contribute to the development of chemoresistance. Because most chemotherapeutic drugs are known to kill cancer cells by inducing apoptosis (47–49), it is likely that TG2 confers drug resistance by up-regulating prosurvival and anti-apoptotic pathways. Indeed, culture of TG2-positive cells on fibronectin-coated surfaces has been shown previously to induce strong activation of the focal adhesion kinase (5, 20, 21, 50), an upstream event that leads to the activation of various downstream antiapoptotic and cell survival signaling pathways (51, 52).

In addition to its role in protecting cells from apoptosis and conferring chemoresistance, TG2 has also been shown to have a proapoptotic role (12–15). The proapoptotic functions of TG2 are linked to the ability of TG2 to irreversibly cross-link proteins in the presence of Ca\textsuperscript{2+}. It is likely that under extremely stressful conditions massive release of Ca\textsuperscript{2+} from intracellular stores or influx of Ca\textsuperscript{2+} from outside the cell leads to activation of TG2 to its cross-linking configuration resulting in post-translational modification of key proteins and onset of apoptosis. Indeed, in the study reported here, we found that TG2-expressing A375 cells exhibited resistance to chemotherapeutic drugs (Fig. 2) but responded well to treatment with the calcium ionophore A23187 (Fig. 3). The A23187-induced cell death of A375 cells was apoptotic (Figs. 4B and 5) and was due to the protein cross-linking activity of TG2 (Fig. 6A). The presence of BPA, a competitive inhibitor of TG2, effectively blocked A23187-induced apoptosis in A375 cells (Fig. 6C).

Based on these results, we propose that high levels of TG2 expression in malignant melanoma cells can confer a drug resistance phenotype by promoting integrin-mediated cell attachment and cell survival signaling pathways. On the other hand, high expression of TG2 in melanoma cells can be exploited as a potential target to kill these hard to treat cancer cells. Our results also indicate that activation of endogenous TG2 could serve as a strategy for inducing apoptosis in malignant melanomas, indicating that high expression of TG2 in melanoma cells can be exploited.

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Figure 10. Fibronectin-mediated attachment and survival of A375 cells is dependent on TG2 expression. Control and siRNA-transfected A375 cells were incubated in serum-free medium on fibronectin-coated plates. After 48-h culture, cells were examined under a light microscope (A) or analyzed for cell viability by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium assay (B).

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