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Original Article

Involvement of extracellular transglutaminase 2 in migration of lung cancer cells

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Transglutaminase 2 (TG2) is a highly complex protein with enzymatic and non-enzymatic functions. It is localized in multiple cellular compartments, inside and outside the cell. Overexpression of TG2 has been implicated in cancer development. However, relatively little attention has been paid to the effects of extracellular TG2. Here, we examined the involvement of extracellular TG2 in cell migration using a lung cancer cell line CL1-0 and its invasive subline CL1-5. Our results showed that CL1-5 exhibits higher levels of TG2 in cell lysates and conditioned medium, when compared with CL1-0. Exogenous application of recombinant TG2 protein to CL1-0 cells substantially augmented migration. Inclusion of TG2 antibody to block extracellular TG2 hampered migration of CL1-5 cells. The stimulatory effect of extracellular TG2 on cell migration was independent of its transamidase activity, as treatment of cells with the TG2 inhibitor Z-DON did not suppress migration. Further examination of the regulatory mechanisms for TG2 expression revealed that the DNA demethylating agent 5-Aza-2'-deoxycystidine increases levels of TG2, but the histone deacetylase inhibitor trichostatin A exerts no effect on CL1-0 cells. Collectively, our results demonstrated the significance of extracellular TG2 in promoting migration of lung cancer cells. Blockade of extracellular TG2 could be a potential strategy for cancer therapy.

Keywords: transglutaminase 2, lung cancer, migration

Introduction

Transglutaminases (TGs; EC 2.3.2.13) are a family of enzymes that catalyze Ca^{+2}-dependent protein crosslinking/transamidation. This occurs through the formation of covalent bonds between the γ-carboxamide group of glutamine and the ε-amine group of lysine residues of proteins [1]. TG2, of the TG superfamily, possesses transamidase and other enzyme activities, including GTPase, ATPase, protein kinase and protein disulphide isomerase activities. It is mainly localized in the cytosol, but is also present in the nucleus, mitochondria, plasma membrane, and extracellular matrix, as well as on the cell surface [2]. Cell surface TG2 associates with fibronectin and integrins to facilitate cell adhesion and migration [3,4]. It also induces integrin clustering, leading to activation of the RhoA pathway [5]. These events do not require the transamidase activity of TG2 [3-5]. Moreover, cell surface TG2 interacts with growth factor receptors, modulating their signaling

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and responses [6,7]. Owing to its multiple functions and localization diversity, TG2 controls numerous cellular responses and aberrant regulation of TG2 has been implicated in several pathological states, such as cancer [1,2].

Accumulating evidence has shown the positive impact of TG2 on cancer development. Overexpression of TG2 in cancer cells promotes metastasis and drug resistance, hallmarks of advanced cancer [8-15]. The mechanism involves TG2-mediated induction of epithelial-mesenchymal transition (EMT) and acquisition of stem cell traits [16,17]. While most studies have focused on the overall effects of TG2, regardless of localization, some reports have revealed the importance of extracellular TG2 in metastasis. In ovarian cancer, abundant TG2 has been detected in malignant ascites [10,18]. Moreover, intraperitoneal administration of recombinant TG2 protein has been found to enhance peritoneal dissemination of ovarian cancer cells [18].

Due to the key role of TG2 in cancer progression, targeting TG2 is a potential strategy for cancer therapy. A number of inhibitors affecting the transamidase or GTPase activity of TG2 or interaction between TG2 and fibronectin have been developed [32-34]. These inhibitors suppress cancer cell migration and invasion and/or enhance the efficacy of chemotherapeutic drugs [11, 13, 32-36]. Alternatively, reducing TG2 expression by small interfering RNA (siRNA) delivered by liposomes has been shown to effectively inhibit metastasis of pancreatic tumors in mice. TG2 also synergizes with gemcitabine to block tumor growth [37].

Lung cancer is the leading cause of cancer-related deaths worldwide [19]. It has been documented that TG2 promotes metastasis and drug resistance in lung cancer cells [13-15, 20]. Interestingly, proteomic analysis of bronchoalveolar lavage fluid has shown that TG2 levels are higher in lung adenocarcinoma samples than in non-cancer control samples [21], implying the potential involvement of extracellular TG2 in lung cancer progression. In this study, we examined this possibility using the lung cancer cell line CL1-0 and its subline CL1-5, which display low and high invasiveness, respectively.

Materials and Methods

Reagents

Recombinant human TG2 protein and mouse monoclonal antibodies to TG2 (clones CUB7402 and CUB7402 + TG100) were purchased from Thermo Fisher Scientific (Fremont, CA). Purified mouse IgG1 (κ isotype control) was obtained from BioLegend (San Diego, CA). ERK antibody was supplied by Santa Cruz Biotechnology (Santa Cruz, CA). TG2 inhibitor Z-DON-Val-Pro-Leu-Ome (Z-DON) was purchased from Zedira (Darmstadt, Germany). Trichostatin A (TSA), 5-aza- 2’-deoxycytidine (5-azadC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Human lung adenocarcinoma cell lines CL1-0 and CL1-5 were kindly provided by Dr. Pan-Chyr Yang (National Taiwan University, Taiwan, ROC). CL1-5, a subline of CL1-0, was isolated from CL1-0 through progressive in vitro invasion screening. CL1-0 and CL1-5 exhibit low and high invasiveness, respectively [22]. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cell viability assay

Cell viability was determined on colorimetric MTT assay. After 1-d exposure to inhibitors, cells were incubated with 1 mg/ml MTT at 37°C for 2.5 h. Formazan salt was then dissolved in 2-propanol and the absorbance at wavelength of 570 nm was measured on an ELISA plate reader.

Cell migration assay

A 24-well modified Boyden chamber with a polycarbonate membrane (8 μm pore size) was used to detect cell migration. Cells were trypsinized, centrifuged, and resuspended in DMEM containing 10% FBS. An equal number of cells (3×10^4 cells/well) were loaded onto the upper chamber. DMEM without FBS was added to the lower chamber. After 24 h incubation, cells were fixed with methanol and stained with Giemsa. Cells on the upper surface

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of the membrane were removed and cells that had migrated to the lower surface of the membrane were counted.

Immunoblotting

Cells were lysed in lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cell lysates containing equal amounts of protein were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (PerkinElmer), and probed with antibodies to TG2 and actin. Proteins were visualized by enhanced chemiluminescence (Cell Signaling).

Statistical analysis

Data are expressed as mean ± S.E.M. of at least three independent experiments. One-way analysis of variance (ANOVA) and Tukey's multiple-comparisons test were used, with $P < 0.05$ considered statistically significant. GraphPad Prism 5 software (GraphPad Software, CA) was used for statistical analyses.

Results

Extracellular TG2 promotes migration of lung cancer cells

Previous studies have revealed a positive role of TG2 in cancer progression, but most have focused on the effects of overall expression of TG2 in cancer cells [1,23]. Given that TG2 is also localized outside cells, we explored the involvement of extracellular TG2 in cell migration using the lung cancer cell line CL1-0 and its subline CL1-5, which display low and high invasiveness, respectively [22]. Amounts of TG2 protein in total cell lysates and medium were higher in CL1-5 than in CL1-0 cells (Figure 1A), closely correlating with their invasive activities. Application of recombinant human TG2 protein to CL1-0 cells effectively augmented cell migration (Figure 1B), suggesting that extracellular TG2 is capable of promoting migration of lung cancer cells. To further demonstrate this, CL1-5 cells were treated with a monoclonal TG2 antibody to block extracellular TG2, as previously described [24]. The result was a reduction in cell migration of 43%. Control IgG exerted no effect (Figure 2). Collectively, these results elucidated the significance of extracellular TG2 in migration of lung cancer cells.

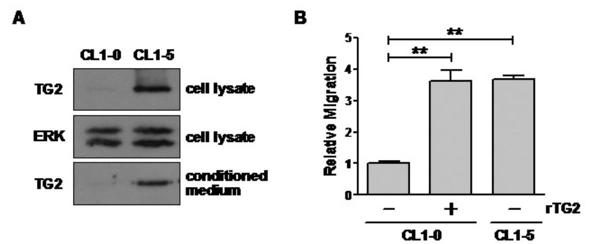

Fig. 1 Recombinant human TG2 protein promotes migration of lung cancer cells. (A) Total cell lysates and culture medium (conditioned medium) collected from CL1-0 and CL1-5 were analyzed by immunoblotting with antibodies to TG2 and ERK. Levels of ERK were used as loading controls. (B) CL1-5 cells were untreated, CL1-0 cells were untreated or treated with 2 μg/ml recombinant TG2 protein, and subjected to migration assay. Data are expressed as fold change relative to untreated CL1-0. ***P* < 0.01, compared with untreated CL1-0.

The transamidase activity of TG2 is not required for migration of lung cancer cells

We have shown that TG2 inhibitors cystamine and monodansylcadaverine (MDC) do not suppress

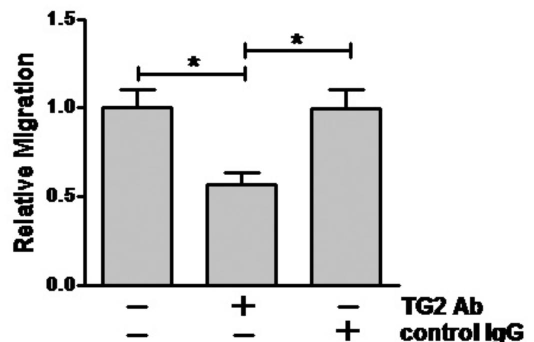

Fig. 2 TG2 antibody hinders migration of lung cancer cells. CL1-5 cells were untreated or treated with a monoclonal TG2 antibody (20 μg/ml) or control IgG (20 μg/ml) and subjected to migration assay. Data are expressed as fold change relative to untreated CL1-5. **P* < 0.05.

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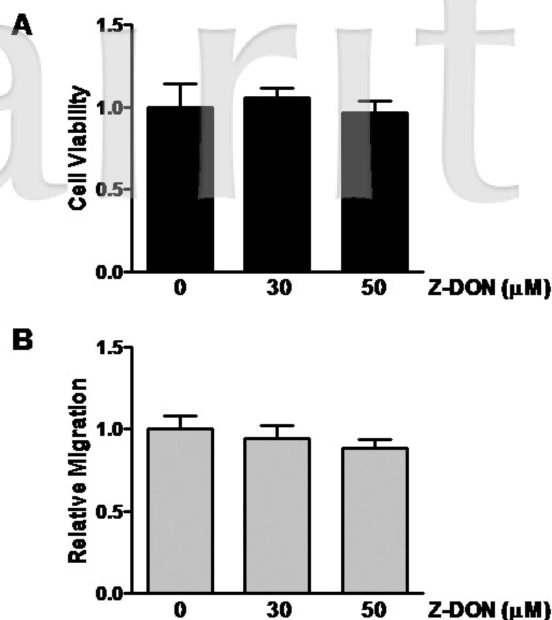

Fig. 3 Inhibition of TG2 activity by Z-DON does not suppress cell migration. (A) CL1-5 cells were untreated or treated with 30 or 50 μM Z-DON for 24 h. Cell viability was determined on MTT assay. (B) Untreated or Z-DON-treated CL1-5 cells were subjected to migration assay. Data are expressed as fold change relative to untreated cells.

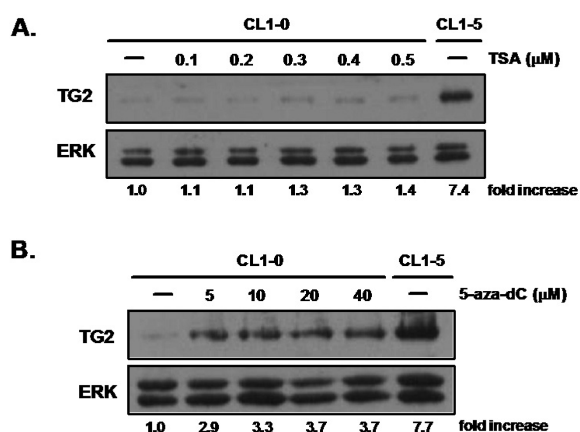

Fig. 4 Inhibition of DNA methylation increases levels of TG2. (A) CL1-5 cells were untreated and CL1-0 cells were treated with 0~0.5 μM TSA. (B) CL1-5 cells were untreated and CL1-0 cells were treated with 0~40 μM AZA. Total cell lysates were analyzed by immunoblotting with antibodies to TG2 and ERK. Levels of ERK were used as loading controls. Data were quantified and normalized to levels of ERK, then expressed as fold increase relative to untreated CL1-0.

migration of CL1-5 cells [38]. Since MDC and cystamine have been reported to display poor selectivity against TG2, we switched to a peptide-like inhibitor Z-DON, which targets the active site cysteine residue and, thus, is far more specific for TG2 [25]. At a concentration of 30 or 50 μM, Z-DON was not toxic to CL1-5 cells and did not inhibit cell migration (Figure 3). As Z-DON is cell permeable at 40 μM, the concentrations we used in this study should be able to inhibit the transamidase activity of intracellular and extracellular TG2 [25]. Thus, regardless of TG2 localization, the stimulatory effect of TG2 on cell migration does not rely on its transamidase activity. This is similar to our previous findings that inactive TG2 is able to promote cell migration [38].

Inhibition of DNA methylation increases TG2 levels in lung cancer cells

Lastly, we explored the regulatory mechanisms of TG2 expression in lung cancer cells. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), as well as epidermal growth factor (EGF), have been shown to induce TG2 expression, but not in CL1-0 cells (data not shown) [1,15]. The involvement of epigenetic mechanisms reported earlier was then examined [2,14]. TSA, an inhibitor of histone deacetylase, did not affect TG2 expression, whereas the DNA demethylating agent 5-aza-dC increased levels of TG2 in CL1-0 cells (Figure 4). These results suggested that the promoter of the TG2 gene (*TGM2*) is hypermethylated in CL1-0 cells, resulting in gene repression. In CL1-5 cells, which exhibit high levels of TG2, inhibition of NF-κB activity by pyrrolidine dithiocarbamate (PDTC) or parthenolide and inhibition of EGF receptor tyrosine kinase activity by AG-1478 did not lower TG2 expression (data not shown).

Discussion

TG2 is a multifunctional protein with localization diversity and its overexpression has been linked to cancer development [1,23]. However, relatively little

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attention has been paid to the effects of extracellular TG2. Here, we show that exogenous application of recombinant TG2 protein augments, whereas inclusion of TG2 antibody blocks, migration of lung cancer cells. The stimulatory effect of TG2 on cell migration is independent of its transamidase activity. Our results revealed the significance of extracellular TG2 in cell migration, which may facilitate dissemination of lung cancer cells

Lacking the signal sequence for the classic endoplasmic reticulum/Golgi-dependent secretory pathway, TG2 is externalized by unconventional mechanisms, such as through microvesicle shedding or targeting of cytoplasmic TG2 to perinuclear recycling endosomes for secretion [26,27]. Although export of TG2 can be regulated, it is generally thought to take place constitutively [2]. Our results were consistent in that they showed that CL1-5 cells express greater amounts of cellular and extracellular TG2 than CL1-0 cells (Figure 1A). We also found that extracellular TG2 stimulates cell migration, as application of recombinant TG2 protein substantially augments migration of CL1-0 cells (Figure 1B). The underlying mechanism might be related to the non-enzymatic scaffolding function of TG2 on the cell surface, i.e., enhanced cell adhesion [28]. One implication of these results is that in the tumor microenvironment, secreted TG2 may exert a pro-invasive effect on neighboring tumor cells, thereby promoting metastasis via the bystander effect. However, extracellular TG2 could become more accessible to therapeutic agents. Administration of TG2 antibody blocks TG2 functions as observed in the present study (Figure 2).

Using the specific TG2 inhibitor Z-DON at different concentrations, we found that extracellular TG2 does not rely on its transamidase activity to stimulate cell migration (Figure 3). This does not oppose the notion that transamidase activity is involved in cancer progression. In the context of tumor tissues, tumor cell-secreted TG2 alters tumor stroma by crosslinking collagen and activating stromal fibroblasts. The resulting microenvironment supports tumor growth and promotes drug resistance [29,30]. Thus, extracellular TG2 on the cell surface and in the extracellular matrix carries out different tasks, culminating in cancer progression.

The abundance of extracellular TG2 is determined, at least in part, by the amount of cellular TG2, which is predominantly regulated at the transcription level [2]. Inflammatory cytokines and growth factors have been shown to induce TG2 expression, and accordingly, various transcription factor-binding sites have been identified in the promoter of the *TGM2* gene, such as the NF-κB-binding site [1,2]. Epigenetic regulation through a control on methylation of the *TG2M* promoter has also been documented [31]. In lung cancer cells, promoter hypermethylation inversely correlates with TG2 expression and inhibition of DNA methylation by 5-aza-dC leads to derepression of the *TG2M* gene [14]. For CL1-0 cells, the most effective way to increase TG2 levels is with 5-aza-dC (Figure 4B). TNF-α, TGF-β and EGF, well-known inducers of TG2 expression, failed to increase TG2 levels (data not shown). These results suggested that the *TGM2* gene is targeted for epigenetic silencing in CL1-0 cells. We also wished to elucidate the mechanism for upregulation of TG2 in CL1-5 cells, but none of the inhibitors we tried (inhibitors for EGF receptor, PDGF receptor, NF-κB, PI3K, MEK, JNK and Src) lowered TG2 expression (data not shown).

In the tumor microenvironment, extracellular TG2 influences not only the cells secreting it, but also bystander cells, such as the surrounding tumor and stromal cells and extracellular matrix. This could lead to tumor growth, metastasis and drug resistance. However, extracellular localization makes TG2 more accessible to therapeutic agents. Blockade of extracellular TG2 by TG2 antibody might be a potential strategy for cancer therapy.

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