Abstract. As an uncommon cancer, mesothelioma is very hard to treat with a low average survival rate owing to its usual late detection and being highly invasive. The link between asbestos exposure and the development of mesothelioma in humans is unequivocal. TGFBI, a secreted protein that is induced by transforming growth factor-β (TGF-β) in various human cell types, has been shown to be associated with tumorigenesis in various types of tumors. It has been demonstrated that TGFBI expression is markedly suppressed in asbestos-induced tumorigenic cells, while an ectopic expression of TGFBI significantly suppresses tumorigenicity and progression in human bronchial epithelial cells. In order to delineate a potential role of TGFBI in mediating the molecular events that occur in mesothelioma tumorigenesis, we generated stable TGFBI knockdown mutants from the mesothelium cell line Met-5A by using an shRNA approach, and secondly created ectopic TGFBI overexpression mutants from the mesothelioma cell line H28 in which TGFBI is absent. We observed that in the absence of TGFBI, the knockdown mesothelial and mesothelioma cell lines exhibited an elevated proliferation rate, enhanced plating efficiency, increased anchorage-independent growth, as well as an increased cellular protein synthesis rate as compared with their respective controls. Furthermore, cell cycle regulatory proteins c-myc/cyclin D1/phosphor-Rb were upregulated; a more active PI3K/Akt/mTOR signaling pathway was also detected in TGFBI-depleted cell lines. These findings suggest that TGFBI may repress mesothelioma tumorigenesis and progression via the PI3K/Akt signaling pathway.

Introduction

Mesothelioma is a rare but deadly form of cancer that is often associated with inhalation of asbestos. It occurs by the transformation of the mesothelium, which is the protective sac covering most of the internal organs of the body. Each year about 2,000-3,000 new cases of mesothelioma are diagnosed in the United States. Although not a common kind of cancer, mesothelioma is very hard to treat. When the symptoms appear and the cancer is diagnosed, the disease is often advanced and the average survival rate is about 1 year. Although the link between asbestos exposure and the cancer is unequivocal, the carcinogenic mechanism is yet largely unknown. SV40 virus has been proposed to be a compounding factor in some mesotheliomas, but the virus exposure cannot be documented in many cases (1). Human mesothelioma cell lines and primary mesothelioma tissues have been examined for alterations in specific oncogenes and tumor suppressors, however, there has been no report on mutation or loss of functions in tumor-related genes such as retinoblastoma (Rb), Wilm's Tumor (WT), Pten, and any of the Ras oncogenes. Mutations in p53 have only been observed in a few cases (2), while Cyclin D1 has been shown to be overexpressed and transforming growth factor (TGF) and other inflammatory cytokines were increased in mesotheliomas (1,2). TGFBI is a secreted protein that is induced by transforming growth factor-β (TGF-β) in various human cell types (3). Mutations or altered expression of this gene is believed to be responsible for the pathogenesis of human corneal dystrophy, angiogenesis, osteogenesis and inflammation (4,5). In addition, the expression of TGFBI is either decreased or absent in 14 human
tumor cell lines as compared to their normal counterparts (6), indicating TGFBI may exert an inhibition on tumorigenesis and/or tumor progression. However, the underlying mechanisms are still unclear.

Over-activation of phosphoinositide 3-kinases (PI3K)/Akt cascade is known for its roles in oncogenic transformation (7,8). Highly activated PI3K has been related to various kinds of human tumors including breast cancer (9), lung cancer (10), melanomas (11), and leukemia (12). Akt is thought to be involved in various cellular processes such as transcrip-

tion, apoptosis and cell cycle progression. Among numerous downstream substrates of Akt, the one that is best-studied is
the serine/threonine kinase mTOR (mammalian target of rapamycin, also termed as TORC1). The activation of mTOR leads to enhanced activity of S6 kinase-1 and phosphorylation of eukaryotic translation initiation factor 4E Binding Proteins 1 (4EBP1), both of which lead to increased protein translation. On the other hand, mTOR also acts as a positive feedback factor to phosphorylate Akt when it binds to Rictor in TORC2 complexes (13). Because of the regulatory role on protein synthesis, which governs cell growth, survival and cellular homeostasis, the PI3K/Akt/mTOR signaling pathway is intimately related with cancer development and progression (14,15).

A recent study in our laboratory showed that TGFBI expression was markedly suppressed in asbestos-induced tumorigenic cells, while a restoring expression of TGFBI gene in asbestos-induced tumorigenic human bronchial epithelial cells significantly inhibited their cell growth and anchorage-independent phenotype in vitro as well as tumorigenicity in vivo (6). Since the PI3K/Akt pathway has been demonstrated to be modulated in various asbestos-treated mesothelial cells (both human and rodent) as well as in human mesothelioma samples (16-19), we postulated that TGFBI might execute a tumor inhibitory function through the PI3K/Akt/mTOR pathway.

Materials and methods

siRNA knock-down of TGFBI in mesothelial cells. The SMARTVector shRNA Lentiviral Particles™ for human TGFBI, SMARTVector Empty Vector Control Particles and SMARTVector non-targeting control particles were produced by Dharmacon (Chicago, IL). Briefly, human mesothelial cell line Met-5A cells were cultured in 96-well plate with Medium 199 (Invitrogen Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) overnight. The medium was then replaced with 80 µl solution containing 3 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) and 20 µl of 5 multiplicity of infection (MOI) SMARTVector shRNA lentiviral particles. The cells were cultured overnight, and then replenished with fresh medium for 72 h. After transduction, cells were trypsinized and plated into 100 mm dish described above containing 0.4 µg/ml puromycin (Sigma-Aldrich). After 2 weeks of culture, successfully transduced colonies were selected and expanded by continuing culture in flask.

Ectopic expression of TGFBI in H28 mesothelioma cells. Cells were purchased from ATCC (Manassas, VA) and transfected with plasmid pRc/CMV2 containing human TGFBI cDNA or the vector alone using calcium phosphate transfection method. G418 (Sigma-Aldrich, 1 mg/ml)-resistant clones were isolated and cell culture supernatant was screened by Western blotting for secreted TGFBI protein. The supernatant was concentrated 10 times with SP Sepharose Fast Flow resin (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) before the assay. TGFBI antibody was purchased from R&D Systems (Minneapolis, MN).

Growth kinetics assay and plating efficiency. Cells were seeded at a density of 1x10^4 in 60 mm diameter dishes from exponentially growing phase. Triplicate dishes from each group were trypsinized and total number of cells per dish was counted by Coulter Counter (Beckman Coulter, Inc. Miami, FL) at indicated time points. Plating efficiency (PE), a measurement of the number of colonies originating from single cells, was used for determination of the effect of TGFBI on cell clonogenic ability. PE is determined by the following formula: PE (%) is equal to the number of colonies counted divided by the number of cells inoculated and multiplied by 100.

Determination of anchorage-independent growth. Cells were plated in triplicate at a density of 1x10^4 cells in 1 ml of 0.35% low melting agarose containing 10% FBS DMEM, and then overlaid on a 0.7% agar base in a 6-well culture plate. After the agar-cell mixture had solidified, 1 ml of 10% FBS DMEM was added on the top. After 3 weeks incubation at 37°C with 5% CO_2, colonies with >30 cells were scored under a light microscope at low magnification.

Immunohistochemical staining. Cells were grown as polarized monolayers on 12 mm tissue culture inserts (Costar). Immunostaining was performed with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Briefly, 4% paraformaldehyde-fixed cells were stained by incubating with the primary antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, 1:100 diluted in 1X PBS with blocking buffer 5% BSA) for 2 h at 37°C. After washing with PBS, biotin-conjugated secondary antibody was applied to the slides for 30 min followed by avidin-biotin- peroxidase complex for 30 min. The slides were then exposed to a reaction solution containing the 3,3'-diaminobenzidine (DAB) for 6 min and washed with distilled water and counterstained with Meyer's hematoxylin for 10 sec. The slides were dehydrated, cleared, and mounted. The slides were examined and images were captured using an Olympus BX60 camera (Olympus, Tokyo, Japan).

Immunoprecipitation (IP) and Western blot analysis. For epidermal growth factor (EGF) stimulation, cells at 80 to 90% confluence were serum starved for up to 24 h and then stimulated at indicated time points. Cells with or without EGF stimulation were rinsed twice with cold PBS and scraped by rubber policeman after adding proper amount of protein lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride). Lysed cells were centrifuged at 12,000 g for 15 min at 4°C, then the supernatant was collected and the protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). For IP, 500 µg cell lysates were incubated with 1st antibody for 2 h, then Protein A/G beads (Santa Cruz Biotechnology) were added and continued to incubate for another hour, then spun down and washed three times with cold lysis buffer. The precipitated beads were added with 2X SDS
loading buffer and boiled for 5 min, prepared for SDS-PAGE. For Western blotting, equal amount of proteins (30 µg) was fractionated by SDS-PAGE and transferred to PVDF membranes under semi-dry condition. Antibodies to EGF receptor, p85, phosphor-AKT, pan-AKT, phosphor-mTOR, mTOR, phosphor-p70S6, p-70S6, phosphor-4EBP1, 4EBP1, and β-actin were obtained from Cell Signaling Technology. Secondary antibodies were from GE Healthcare (Piscataway, NJ). Signals were detected using ECL (Pierce, Rockford, IL).

Statistical analysis. Data statistical analysis was carried out using Student's t-test. Differences between means are regarded as significant at \( p \leq 0.05 \).

Results

The knockdown of TGFBI in mesothelial cell line Met-5A and the ectopic expression of TGFBI in mesothelioma cell line H28. The TGFBI knockdown cell lines were generated by introducing the SMARTvector shRNA Lentiviral Particles™ of human TGFBI into Met-5A cell line which expresses endogenous TGFBI. As shown in Fig. 1A, in the three TGFBI knockdown cell lines, designated as 5AT1, 5AT2 and 5AT3, much lower TGFBI protein levels were detected compared with those in the non-transfected (5ATN) or scrambled RNA transfected cell lines (5ATE). Meanwhile, the mesothelioma cell line H28 which does not express endogenous TGFBI, was transfected with ectopic TGFBI. Three TGFBI-transfected cell lines, H28T4, H28T6 and H28T7, exhibit substantial amount of TGFBI (Fig. 1B). We then chose 5AT1, which has undetectable TGFBI and the scrambled RNA transfected cell line 5ATE for most of the studies in this report; so as to H28V, the empty vector transfected cell line, and TGFBI expressing H28T7 were selected in the parallel study. For confirming the consistency or minimal variation among different clones, additional clones were included in the study of some key characteristics such as growth kinetics, plating efficiency, and anchorage-independent growth.

Cell growth was enhanced in TGFBI knockdown Met-5A cells but suppressed in TGFBI ectopically expressing H28 cells. To evaluate the effect of TGFBI on cell survival and proliferation, we assessed cell growth rate in certain number of cell lines we generated. Although there were only marginal differences among these cell lines in the first three days after plating cells, 5AT1 and 5AT2 cells which had very close growth kinetics, among these cell lines in the first three days after plating cells, exhibited a notably enhanced grow rate versus 5ATE and 5ATN after day 4, while the later two cell lines exhibited strong similarities, the trend was maintained till day 9, when cell growth and survival ability, plating efficiency, and anchorage-independent growth.
Concerning how TGFBI achieved the above effects, the expression profile of several cell cycle regulatory proteins was examined. As shown in Fig. 2C, the expressions of c-myc, cyclin-D1 and phosphor-Rb were all up-regulated in both 5AT1 and H28V, compared with their corresponding TGFBI-expressing controls 5ATE and H28T7, respectively; quantitative data show 2-4-fold increases in Fig. 2D. The results suggest an inhibitory role of TGFBI on cell growth, which might be related to its regulation of cell cycle components.

After establishing the suppressive effects of TGFBI on cell growth, we further asked if it exhibits a similar effect on neoplastic phenotype. As shown in Fig. 2E, ablation of TGFBI significantly increased the anchorage-independent growth in 5AT1 and 5AT2, versus 5ATE and 5ATN, respectively; quantitative data show 2-4-fold increases in Fig. 2D. The results suggest an inhibitory role of TGFBI on cell growth, which might be related to its regulation of cell cycle components.

The absence of TGFBI potentiates EGF-induced PI3K/Akt/mTOR pathway. Accumulating evidence demonstrated that PI3K/Akt cascade is modulated in human and rodent mesothelial cells after being treated with asbestos, as well as in human mesothelioma samples (16-19). We, therefore, wanted to see if the increased cellular activities depicted above in TGFBI deficient cells are related to the alteration of this pathway. First we assessed the phosphorylation status of AKT after epidermal growth factor (EGF) induction, a potent mitogen often used to induce AKT activation. As indicated in Fig. 3A, though both 5ATE and 5AT1 cells showed an induction in phosphorylation after EGF stimulation, 5AT1 had a much stronger response compared with that of 5ATE cells. Similarly, H28T7 showed a slower and less dramatic response in AKT phosphorylation upon EGF stimulation than H28V (Fig. 3B). These data strongly indicate that the absence of TGFBI potentiates the activation of Akt upon growth factor stimulation.

mTOR is an evolutionarily conserved checkpoint protein kinase that serves as a major effector of cell growth and proliferation mediated by the PI3K/Akt pathway (20). Therefore, we next examined the phosphorylation status of Ser2448 in mTOR, an indicator of its activation, in these four cell lines. As shown in Fig. 3C, there was a significant increase in mTOR Ser2448 phosphorylation in 5AT1 cells upon EGF stimulation. The response was prompt, peaked at 5 min and then gradually decreased; whereas, only a marginal increase in mTOR Ser2448 phosphorylation was observed in 5ATE cells. Consistently, a similar effect with slightly different pattern on mTOR phosphorylation was documented in H28V versus H28T7 cells (Fig. 3D).
Activation of mTOR downstream targets in the absence of TGFBI. S6K1 and 4EBP1 are well-characterized downstream targets of mTOR, both of which are important regulators of mRNA translation and protein synthesis (21,22). Because of the direct link between mTOR and S6K1 and 4E-BP1, the phosphorylation status of these two proteins is widely used as a surrogate measuring mTOR activity (23). Therefore, after confirming the regulation of mTOR activity by TGFBI, the phosphorylation status of S6K1 and 4EBP1 was assessed. As predicted, 5AT1 cells, which TGFBI is deficient, possessed a significantly higher S6K phosphorylation upon EGF stimulation which sustained till 40 min, as opposed to 5ATE cells, which exhibited only minimal response (Fig. 4A); likewise, the phosphorylation of 4EBP1 was shown in almost the same pattern as that of S6K1 in 5AT1/5ATE cells (Fig. 4A). In H28V cells, both S6K and 4EBP1 are markedly phosphorylated in response to EGF treatment, higher than in H28T7 cells, with S6K exhibiting a more transient response as compared with 5AT1; while that of 4EBP1 showed a similar pattern in these two sets of cells (Fig. 4B).

In order to evaluate the role of TGFBI under a general, non-stimulated culture condition, we examined the phosphorylation status of the components in the PI3K/Akt/mTOR pathway in all four cell lines without external stimulation. As shown in Fig. 5A and B, the phosphorylations of Akt, mTOR, S6K and 4EBP1 were all enhanced in TGFBI-deficient cell lines versus TGFBI-expressing cell lines, i.e., 5AT1 versus 5ATE, and H28V versus H28T7. The results above unequivocally support the assertion that TGFBI has a suppressive effect on PI3K/Akt/mTOR signaling pathway.

Protein synthesis was enhanced in TGFBI-deficient cells. After establishing that Akt/mTOR pathway was up-regulated in TGFBI-deficient cells, we asked whether the absence of TGFBI indeed affected protein synthesis in cell culture, which would be ultimately translated into a perceived functional molecule. As shown in Fig. 6, both 5AT1 and H28V, cells without TGFBI have significantly enhanced protein synthesis rate compared with their TGFBI-expressing counterparts, establishing that TGFBI plays a suppressive role in protein synthesis.

The absence of TGFBI leads to enhanced binding of P85 of P13K to peptide growth factor receptor. Since TGFBI is an extracellular matrix protein (ECM), it is imperative to elucidate how it transduces signal into cells, thereby regulating PI3K/AKT/mTOR and ultimately exerts its suppression on tumorigenesis. In integrin signaling, TGFBI has been shown to be a ‘linker’ participating in the interaction between ECM and integrins (24). Under non-stimulated condition, the p85 regulatory subunit of PI3K serves to stabilize and inactivate the p110 catalytic subunit. With the stimulation of growth factor receptor, p85 binds to the phosphorylated tyrosine sites on the cytoplasmic domain of the activated receptor through its SH2 domain and
then releases the inhibition of PI3K (25). To examine whether TGFBI affects the complex formation between growth factor receptor and p85 subunit, we used immunoprecipitation to detect it. As shown in Fig. 7, in 5AT1 cells, the binding of p85 with EGF receptor showed an increased intensity as compared with 5ATE, culminated at 10 min. Similar result was obtained in H28V/H28T7 cells (data not shown).

Discussion

Malignant pleural mesotheliomas (MPM) are very aggressive tumors with an extremely poor prognosis with median survival duration of 1-year and a 5-year survival rates of ≤1% (26). MPM causes around 15,000-20,000 deaths annually worldwide (27) and the incidence of this disease is steadily increasing throughout most part of the world, as a result of widespread exposure to asbestos during past decades (27-31).

Various evidence has been linked to the development of MPM. For instance, p53 has been shown to be modulated in some cases (2). Enhanced production of some growth factors and cytokines, such as platelet derived growth factor (PDGF), transforming growth factor (TGF), insulin growth factor (IGF), fibroblast growth factors (FGF), vascular endothelial growth factors (VEGFs), and interleukin 6 (IL-6), IL-8, possibly resulting from the activation of certain oncogenes, was found in some malignant mesothelioma cell lines (32). However, the complete pathophysiology involved in the development of MPM is far from clear.

TGFBI, otherwise known as Betaig-h3, was reported to be involved in the suppression of asbestos-induced tumorigenesis (6). A recent study in our group demonstrated that TGFBI expression was down-regulated in asbestos transformed human bronchial epithelial cells, while restoring TGFBI expression significantly suppressed the cell growth and anchorage independent phenotype in vitro and tumor incidence in vivo (6). More than 10 years ago, TGFBI was reported in the regulation of tumorigenesis when CHO cells expressing the protein had a reduced ability to form tumors in nude mice (33); later, FAS1 domains of TGFBI have been demonstrated to inhibit tumor
angiogenesis and also tumor growth, as well as promoting apoptosis, evidence which is consistent with a tumor suppressor role of TGFBI (34). In addition, our group has shown that: a) many tumor cell lines have decreased expression of TGFBI and b) tumorigenic cell lines exogenously expressing TGFBI have decreased tumorigenesis in vitro and in vivo (35,36).

Since tumorigenesis is a process spanning from a non-malignant state to a malignant state, two types of cell lines - one derived from a non-malignant mesothelial cell line Met5A, the other one derived from the mesothelioma cell line H28, were engineered for the purpose of this study.

Tumorigenesis is a multistage process; changes in cellular growth pattern are often accompanied in this process (37). By generating a TGFBI knockdown cell line 5AT1 and an ectopically TGFBI-expressing cell line H28T7 from each of their parental cell lines, we demonstrated that TGFBI displays an inhibitory role in cell growth, suggesting that this phenotype may represent an early event of transformation exerted by depletion of TGFBI. The concurrent increased plating efficiency indicated there was a portion of highly proliferating cells which acquired maximal activation) versus non-stimulated random growth condition (less activated but more physiological), which well-explained the increased cell growth kinetics in TGFBI-deficient cells shown in Fig. 1, and was also consistent with a separate study in our group that TGFBI-expression cells had an elevated G1 percentage and transiently increased p21 and p53 expression (unpublished data).

In vitro anchorage-independent growth usually correlates with tumorigenesis in many cell types; in normal adherent type of cells, when detached from substrates they will eventually undergo anoikis (39). In the absence of TGFBI, we observed increased levels of anchorage-independent growth compared to controls (Fig. 2D), which corresponds to our previous observations in different type of tumorigenic cells expressing exogenous TGFBI (6), strongly suggesting that loss of TGFBI expression may adapt the cells to receive survival signals irrespective of the unfavorable environment, and potentially gain the ability to undergo tumorogenic transformation.

The PI3K/Akt signaling pathway is well known for its crucial role in cell growth and survival. Upon activation, PI3K catalyzes the phosphorylation of phosphoinositides and generates phosphatidylinositol-3,4,5-triphosphate [PI(3,4,5)P3] and phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2], both of which are biologically active (6). PI(3,4,5)P3 binds to the pleckstrin homology (PH) domains of the downstream serine/threonine kinase Akt, causing its translocation to the cell membrane, where it executes its functions (14). PI3K/Akt and its downstream effector mTOR have been shown to be modulated in various asbestos-treated mesothelial cells, both human and rodent, as well as in human mesothelioma samples (16-19). Mesothelioma cells treated with PI3K inhibitor LY294002 was found to have a synergistic effect on inhibiting cell proliferation and inducing apoptosis when combined with cisplatin, a cell division interfering drug (16). Treatment of mesothelioma cell lines with the mTOR inhibitor rapamycin could cause growth arrest in G1 phase (16).

Therefore we postulated that TGFBI might execute its inhibitory function through modulating the PI3K/Akt/mTOR pathway. Indeed, under EGF-stimulated condition (PI3K/AKT acquire maximal activation) versus non-stimulated random growth condition (less activated but more physiological), or, in tumor cell lines versus non tumor cell lines, the phosphorylation of AKT and mTOR in the absence of TGFBI was substantially increased (Figs. 3 and 5), which is in accordance with the concomitant phenotypic changes.
As downstream target of mTOR, S6K1 enhances protein synthesis upon activation by facilitating PDCD4 degradation via phosphorylation, which in turn releases elf4A helicase from the wind-up secondary structure in mRNA 5' untranslated region (21-23). In addition, S6K1 also phosphorylates the ribosomal protein 4EBP1, releases it from hindering the elongation initiation factor 4E (eIF4E) and thus promotes mRNA translation. Through controlling cellular protein synthesis, S6K1 and 4EBP1 affect a wide variety of biological functions, herein, tumorigenesis - a process with constantly demanding protein synthesis. In TGFB-deficient cells, regardless of tumor or non-tumor cell lines, on EGF stimulation or not, a significantly higher phosphorylation of S6K and 4EBP1 were documented in this study, which also dictates the enhanced protein synthesis, as shown in Figs. 4, 5 and 6.

Under non-stimulated conditions, the p85 regulatory subunit of PI3K serves to stabilize and inactivate the p110 catalytic subunit. With growth factor receptor stimulation, p85 binds to the phosphorylated tyrosine sites on the cytoplasmic domain of the activated receptor through its SH2 domain and then releases the inhibition of PI3K (25). As an extracellular molecule, how does TGFB1 send transmembrane signal to intracellular compartments thereby regulating PI3K/AKT? Our data showed that in the absence of TGFB1, the interaction between p85 and EGF receptor was increased (Fig. 7), which led to enhanced PI3K activation. We therefore hypothesize that without the check of TGFB1, which may bind to the extracellular domain of the receptor, an autophosphorylation of the unleashed growth factor receptor would be enhanced, which results in a more activated downstream signaling pathway and associated biological functions. This hypothesis remains to be investigated.

In this study, we demonstrated that TGFB1 suppressed many cancer associated phenotypes, which is consistent with previous findings of our group and others that TGFB1 displayed a tumor suppressor function (6,40). However, recent studies suggested that TGFB1 expression actually increased the metastatic ability of a colon cancer and ovarian cancer cell line (41,42). Furthermore, it has been found that TGFB1 expression increases in some cancer types (41). It may need to be addressed whether this is a cell type-specific phenotype, in which TGFB1 functions differently in a specific scenario; if so, it might be related to the pattern of TGFB1's interaction with different types of receptors other than growth factor receptors, like integrins, etc. (5). Nevertheless, our findings presented here unequivocally indicate that TGFB1 suppresses mesothelioma progression through Akt/mTOR pathway. These results suggest that TGFB1 may be a promising target in the designing of future anti-mesothelioma therapeutics.

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