TNF-α Induces Epithelial–Mesenchymal Transition of Renal Cell Carcinoma Cells via a GSK3β-Dependent Mechanism

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Abstract

TNF-α is a cytokine with antitumorigenic property. In contrast, low dose, chronic TNF-α production by tumor cells or stromal cells may promote tumor growth and metastasis. Serum levels of TNF-α are significantly elevated in renal cell carcinoma (RCC) patients. Here, we showed that TNF-α induced epithelial–mesenchymal transition (EMT) and promoted tumorigenicity of RCC by repressing E-cadherin, upregulating vimentin, activating MMP9, and invasion activities. In addition, TNF-α treatment inhibited glycogen synthase kinase 3β (GSK-3β) activity through serine-9 phosphorylation mediated by the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway in RCC cells. Inhibition of PI3K/AKT by LY294002 reactivated GSK-3β and suppressed the TNF-α–induced EMT of RCC cells. Inactivation of GSK-3β by LiCl significantly increased MMP9 activity and EMT of RCC cells. Activation of GSK-3β by transduction of constitutively active GSK-3β into RCC cells suppressed TNF-α–mediated anchorage-independent growth in soft agar and tumorigenicity in nude mice. Overexpression of a kinase-deficient GSK-3β, in contrast, potentiated EMT, anchorage-independent growth and drastically enhanced tumorigenicity in vivo. Most importantly, a 15-fold inactivation of GSK-3β activity, 3-fold decrease of E-cadherin, and 2-fold increase of vimentin were observed in human RCC tumor tissues. These results indicated that inactivation of GSK-3β plays a pivotal role in the TNF-α–mediated tumorigenesis of RCC. Mol Cancer Res; 10(8); 1109–19. © 2012 AACR.

Introduction

Renal cell carcinoma (RCC) is the tenth most common cause of cancer-related deaths worldwide (1). Although surgery is often curative, 30% of patients will present with metastases at the time of initial diagnosis (1). The 5-year survival rate is only 5% in metastatic RCC as advanced RCC is resistant to chemotherapy and radiotherapy (2, 3). Previous studies indicated immunotherapy is relatively effective against RCC (2, 3). However, the response rate is only 15% to 20% (1–3). Therefore, defining the factors involved in disease progression and metastasis will provide novel molecular targets for the development of effective therapies.

TNF-α with antitumorigenic property is mainly produced by activated macrophages. In animals, high doses of TNF-α can lead to anticancer effects via activation of T-cell–mediated immunity and selective destruction of blood vessels. In contrast, low dose, chronic TNF-α produced from a number of cancer cells, including RCC, and stromal cells may promote tumor growth and metastasis (4–6). Furthermore, the presence of TNF-α in tumors is associated with poor prognosis, hormone resistance, and cachexia (4–6). In RCC, inflammatory reactions are correlated with a poor patient prognosis. The serum levels of TNF-α are significantly higher and correlated with tumor size in RCC (7, 8). In addition, infliximab, a chimeric anti-human TNF-α antibody, was effective in the therapy of RCC patients in 2 phase II studies (8). However, the detailed molecular mechanism of TNF-α involvement in RCC tumor progression remains unknown.

Glycogen synthase kinase 3β (GSK-3β) is a multifunctional serine/threonine kinase and a critical regulator in glycogen metabolism. GSK-3β is also involved in the regulation of cell mobility, cell proliferation, and apoptosis (9–11). Unlike most protein kinases, GSK-3β is constitutively active in resting cells. GSK-3β activity is rapidly and transiently downregulated by phosphorylation at Ser9 when stimulated by external signals. Several kinases are capable of inactivating GSK-3β, for example, protein kinase B (Akt) and protein kinase A (9–11). Because GSK-3β negatively regulates many proto-oncogenes and cell cycle regulators,
it has been found to act as a tumor suppressor in nonmelanoma skin cancers, mammary tumors, and gastric cancers (12–14). However, the involvement of GSK-3β in tumor development is contradictory (11, 15). Some studies suggest that GSK-3β may promote tumorigenesis in human ovarian, colon, and pancreatic carcinomas. The role of GSK-3β during TNF-α–promoted tumorigenesis of RCC remains unclear.

In this study, we found that TNF-α induced epithelial–mesenchymal transition (EMT) and promoted tumorigenicity of RCC by repressing E-cadherin, upregulating vimentin expression, activating matrix metalloproteinase 9 (MMP9), and increasing invasion activities. Inactivation of GSK-3β is crucial for TNF-α–enhanced EMT and tumorigenicity in RCC. Moreover, inactivated GSK-3β and promoted EMT were observed in human RCC tumor tissues. Constitutive activation of GSK-3β in RCC led to retardation of the tumor growth in mice. Our data suggest that activation of GSK-3β may be a novel molecular target for therapy of RCC.

Materials and Methods

Cell lines and mice

Human RCC cell lines were obtained from American Type Culture Collection (ATCC) and Bioresource Collection and Research Center (BCRC) in 2009. 786-O and Caki-1 (ATCC HTB-46 and ATCC CRL-1932) cells were maintained in RPMI medium (HyClone Laboratories) supplemented with 10% FBS (HyClone Laboratories) and 1% penicillin–streptomycin (P-S; Gibco BRL Life Technologies). ACHN (ATCC CRL-1611) cells were maintained in modified Eagle’s medium (HyClone Laboratories) supplemented with 10% FBS (HyClone Laboratories) and 1% penicillin–streptomycin (P-S; Gibco BRL Life Technologies). A498 cells (ATCC HTB-44) were maintained in modified Eagle’s medium supplemented with 10% FBS, 1% P-S, and 1 mmol/L sodium pyruvate (Gibco BRL Life Technologies). A498 cells (ATCC HTB-44) were maintained in modified Eagle’s medium supplemented with 10% FBS, 1% P-S, 1 mmol/L sodium pyruvate. All cells were cultured in a humidified incubator containing 5% CO2 at 37°C.

Human tumor biopsies

Tumor and normal corresponding tissues of 8 patients with RCC (Supplementary Table S1) were obtained from the Division of Urology, Department of Surgery, Tri-Service General Hospital and National Defense Medical Center (Taipei, Taiwan, ROC). Informed consent was obtained from all patients (IRB 096-05-003). Immediately after surgical resection, tissues were fresh frozen within 1 hour and kept in liquid nitrogen until protein analysis.

Plasmids, antibodies, and reagents

Plasmid pcDNA and the constitutively inactive (HA_CT_Hu_GSK3βb_pcDNA3_ K-A cor) and active (pcDNA3GSK3betaCTA9Good) mutants of GSK-3β plasmids (13) were kindly provided by Dr. J.R. Woodgett (Department of Medical Biophysics, University of Toronto, Canada). Recombinant human TNF-α was purchased from Invitrogen. PI3 kinase inhibitor (LY294002) was obtained from Calbiochem. GSK3β inhibitor (LiCl), anti-vimentin (V9), and anti-β-actin (AC-15) antibodies were purchased from Sigma-Aldrich. Anti-E-cadherin antibody was purchased from BD Bioscience. Anti-phospho Akt (Ser 473), anti-HA (F-7), and anti-TNFR1 (G-2) antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho GSK-3α/β (Ser21/9, inactivating residues) antibody was purchased from Cell Signaling Technology. Anti-GSK-3α/β (4G-1E) antibody was obtained from Upstate Biotechnology. Horse-radish peroxidase (HRP)-conjugated anti-mouse IgG antibody was purchased from Jackson ImmunoResearch Lab. Inc. HRP-conjugated anti-rabbit IgG antibody was purchased from Amersham Biosciences. Alexa Fluor 488–conjugated anti-mouse IgG antibody was obtained from Invitrogen, and the jetPEI PolyPlus transfection reagent from Poster.

Analysis of gene expression

After serum starvation (0.5% FBS) for 16 to 18 hours, tumor cells (2 × 10^5/mL) were treated with or without TNF-α (50 ng/mL) for 24 hours. Total cellular RNA was extracted using the Ultraspec RNA isolation system (BIO-TEC BX Lab., Inc.) or RNeasy kit (Qiagen). Each extracted RNA sample (5 μg) was reversely transcribed into cDNA, followed by the manufacturer’s protocol for reverse transcriptase PCR (RT-PCR; Promega). The amplification was carried out for 30 cycles. The forward and reverse primers were synthesized by Sigma-Prologio (Supplementary Table S2). The transcript levels of the different genes were quantified by quantitative RT-PCR (qRT-PCR) with specific primers (Supplementary Table S2) and SYBR Green I protocol (Bio-Rad) in an ABI7700 System (Applied Biosystems). All values were normalized against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA of human cell lines. The gene expression as fold change was plotted relative to the level of untreated cells or TNF-α–treated cells for 0 hours.

Western blot

After serum starvation (0.5% FBS) for 16 to 18 hours, RCC cells (5 × 10^5/mL or 2 × 10^5/mL) were treated with or without TNF-α (50 ng/mL) for 1 hour to detect the transcriptional factors or for 4 days to examine the EMT markers. Total cellular proteins from transfectants and treated cells were extracted in the lysis buffer [50 mmol/L Tris (PH 7.4), 10% glycerol, 150 mmol/L NaCl, 1% Triton-X, 1.5 mmol/L MgCl2, 50 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L PMSF, 1 mmol/L EDTA,
and 2 µg/mL aprotinin]. Equal amount of protein samples (60 µg from renal cancer cell lines or 20 µg from clinical specimens) were loaded onto 10% SDS-PAGE and transferred to nitrocellulose (Millipore) filters for Western blot analysis.

**Immunofluorescence assay**

After serum starvation (0.5% FBS) for 16 to 18 hours, RCC cells (5 x 10^4/mL) were incubated with or without TNF-α (50 ng/mL) for 4 days. Cells were then fixed in PBS containing 4% formaldehyde and 400 mmol/L sucrose followed by permeabilization in 1% Triton X-100. After washing, cells were blocked in 5% BSA and incubated with anti-vimentin antibodies, and then incubated with Alexa Fluor 488-conjugated anti-mouse IgG antibodies. The cells were then stained for nuclei and F-actin with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and rhodamine-phalloidin (Invitrogen). Cells were mounted in Immunofluore mounting medium (Dako), and images were captured using an Olympus FV1000 confocal microscope (16).

**Invasion and migration assays**

Transwell inserts (8-µm pore; Costar) were placed into the wells. In invasion assay, Matrigels (BD Biosciences) were hydrated with 10% FBS-RPMI. A total of 3 x 10^4 cell suspension was added to the upper chamber, and 10% FBS-RPMI and placed in the upper chamber, and 10% FBS-RPMI was added to the lower chamber. At day 21, cells were stained for examination of colony growth. Colonies more than 50 cells were counted and the results were expressed as mean number of colonies ± SD.

**Anchorage-independent growth assay**

Approximately 3 x 10^3 cells starved and with or without TNF-α-treated cells as the immunofluorescence assay above were resuspended in 0.5% FBS-RPMI and placed in the upper chamber, and 10% FBS-RPMI was added to the lower chamber. After a 20-hour incubation at 37°C in a humidified 5% CO₂ atmosphere, the cells that had invaded to the other side of the membrane were fixed with methanol and the Matrigel, and noninvaded cells were mechanically removed with a cotton swab. The adherent cells on the membrane were stained with Li-F stain (MUTO) and examined under microscopy at 100 x magnification. In migration assay, the Transwell inserts were not coated with Matrigel, and the other procedures were the same as the invasion assay protocol (16).

**Gelatin zymography**

After serum starvation (0.5% FBS) for 16 to 18 hours, RCC cells (5 x 10^4/mL) were treated with or without TNF-α (50 ng/mL) for 4 days. Cells (1 x 10^5/mL) were then incubated for 2 days and then cultured in serum-free medium for 1 day. Culture supernatants were collected and concentrated with Vivaspin 6 centrifugal concentrators (Vivasience). Concentrated supernatants (20 µg) were loaded for electrophoresis under nonreducing conditions on 10% SDS-polyacrylamide gels containing 1 mg/mL gelatin, as previously described (17). After electrophoresis, the gels were washed in 2.5% Triton X-100 and then incubated for 20 to 22 hours at 37°C in the development buffer containing 0.01 mol/L CaCl₂ and 0.05 mol/L Tris-HCl (pH 8.0). The zymographic gels were fixed in 50% methanol and 10% acetic acid and stained with 0.5% Coomassie brilliant blue R-250, and then destained in 10% acetic acid. Proteolytic activity was detected as clear bands (zones of gelatin degradation) against the blue background of stained gelatin. The gels were scanned with a Laser Scanning Densitometer and signals were quantified with ImageQuant 5.2 software (Amersham Bioscience).

**In vivo tumor growth**

After serum starvation (0.5% FBS) for 16 to 18 hours, RCC cells (5 x 10^4/mL) were incubated with or without TNF-α (50 ng/mL) for 6 days and then dissociated from monolayer cultures by trypsinization. Cells were counted and centrifuged at 1,200 rpm for 5 minutes and resuspended in PBS. An aliquot of cells (5 x 10^5/100 µL of PBS) that were treated with or without TNF-α (50 ng/mL) was directly subcutaneously injected to 6 to 8 weeks old male NOD/scid (H-2b) mice. Tumor growth was measured with a caliper and calculated as length x width x height (in mm³) at intervals of 3 days (16). In addition, tumor tissue was collected from each group and analyzed for proteins expression by Western blotting.

**Immunohistochemistry**

The sections of formalin-fixed and paraffin-embedded specimens were autoclaved in DAKO REAL target retrieval solution (Dako) at 121°C for 10 minutes. To remove the endogenous peroxidases activity, the sections were immersed in 3% H₂O₂ for 10 minutes. The samples were subsequently blocked with Ultra V Block (Thermo scientific) for 5 minutes and incubated with primary antibodies at 4°C overnight. Detection of immunostaining were determined by UltraVision Quanto Detection System HRP DAB kit (Thermo scientific) or Zymed AEC substrate kit (Zymed Laboratories) according to the manufacturer’s protocol. Finally, the sections were counterstained with hematoxylin, and photomicrographs were taken in bright field setting using Olympus BX51 microscope.

**Statistics**

Data were expressed as mean ± SD and statistical significance was assessed by the Student t test.

**Results**

**TNF-α stimulated cellular transformation of RCC cells**

To test whether stimulation of TNF-α has an effect on the tumor progression of RCC, we first examined the protein levels of TNF-α receptor (TNFRI) in human 786-O, Caki, ACHN, and A498 RCC cells and the effect of TNF-α on...
growth of these cells. Western blotting results indicated that the TNFRI was expressed among the RCC cells (Fig. 1A, top). Although TNF-α did not induce cellular proliferation or death of RCC cells (data not shown), the treatment of 786-O cells resulted in a morphologic change from predominantly epithelial to spindle fibroblast-like shape in 786-O cells (Fig. 1B). Upon close examination with phalloidin staining, F-actin-positive membrane protrusions were significantly increased in the TNF-α–treated cells (Fig. 1B). The level of vimentin, a mesenchymal marker, was dramatically increased in the TNF-α–treated 786-O cells (Fig. 1B). In Western blot analysis, vimentin levels were obviously increased in response to TNF-α, whereas levels of epithelial cell marker, E-cadherin, were decreased in 786-O, Caki-1, and A498 cells (Fig. 1A, bottom). In addition, the mRNA levels of the transcriptional repressors of E-cadherin, ZEB1, and Slug, but not Snail, were enhanced by TNF-α treatment (Supplementary Fig. S1A, bottom), which may contribute to E-cadherin downregulation (18). We further found that TNF-α induced high levels of MMP9 but not MMP2 expression in a time-dependent manner in 786-O cells (Supplementary Fig. S1A, top). These results showed that TNF-α plays an important role in the promotion of EMT in RCC.

TNF-α enhanced migration/invasion and tumorigenicity of RCC cells

Because the phenotypic result of EMT as well as enhanced MMP9 expression may lead to promotion of cellular mobility (18, 19), the cellular migration and invasive activities of RCC upon TNF-α stimulation were investigated. TNF-α treatment induced a 2-fold increase in the migration and invasion abilities of RCC cells (Fig. 1C). The effect of TNF-α on the transformation of RCC cells was further detected by anchorage-independent growth in soft agar; TNF-α promoted the colony formation of all 3 RCC cells, 786-O, Caki-1, and A498 (Fig. 1D). We then further investigated the effect of TNF-α on tumorigenicity in vivo. We subcutaneously inoculated TNF-α–treated 786-O cells into nude mice and found that the tumor growth rate was enhanced in mice bearing TNF-α–treated 786-O cells (1,010 mm3/60 d) compared with control cells (625 mm3/60 d; Supplementary Fig. S1B). Thus TNF-α enhanced the mobility of RCC cells in vitro and promoted their tumorigenicity in vivo.

TNF-α–mediated changes of E-cadherin and vimentin were via the PI3K/Akt and GSK-3β pathways in RCC cells

Previous study showed that the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway was activated to inhibit GSK-3 kinase in RCC cells (20). GSK-3 kinase has been reported to be involved in the regulation of EMT (11). To further investigate the underlying mechanisms involved in TNF-α–mediated EMT in RCC cells, we analyzed whether TNF-α has an effect on the PI3K/Akt and GSK-3 signaling pathways. TNF-α treatment activated Akt in a time-dependent manner, which was concomitant with inactivation of GSK-3 by phosphorylation of its serine-9 (Ser9) residue in 786-O cells (Fig. 2A). To ascertain whether the PI3K/Akt pathway was involved in the TNF-α–mediated EMT of RCC, we examined the effect of LY294002, a PI3K inhibitor, on the expression of E-cadherin and vimentin. LY294002 pretreatment inhibited TNF-α–induced phosphorylation of Akt and GSK-3β (Data not shown) and reversed the upregulation of vimentin and downregulation of E-cadherin mediated by TNF-α in
both RCC cells, respectively (Fig. 2B, top). In addition, pretreatment with LY294002 resulted in downregulation of TNF-α–induced expressions of ZEB1 and Slug transcripts in 786-O cells (Fig. 2C). These data indicated that inhibition of PI3K/Akt and GSK-3β signaling was required for regulation of EMT in RCC cells.

To further evaluate the role of GSK-3β inactivation on the TNF-α–induced EMT, treatment with lithium chloride (LiCl), a GSK-3β inhibitor, with or without TNF-α resulted in equal downregulation of E-cadherin and upregulation of vimentin in both RCC cells (Fig. 2B, bottom). The high expressions of vimentin and F-actin were also observed in 786-O cells treated with LiCl by immunofluorescence analysis (Fig. 2E). LiCl treatment with or without TNF-α enhanced the expression of ZEB1 and Slug transcripts (Fig. 2D). However, LiCl pretreatment did not have any effect on upstream Akt activation by TNF-α (data not shown). These results suggested that inactivation of GSK-3β was the key molecule responsible for TNF-α–mediated EMT in RCC cells.

The roles of PI3K/Akt and GSK-3β in TNF-α–induced EMT of RCC cells were investigated by treating 786-O and Caki-1 cells with various agents and soft agar assays carried out. The colony formation of both RCC cells was significantly suppressed by LY294002 even in the presence of TNF-α. In contrast, LiCl treatment enhanced colony formation despite the lack of TNF-α stimulation (Fig. 3A). We further examined the effects of PI3K/Akt and GSK-3β on TNF-α–mediated MMP9 expression and activity in RCC cells. TNF-α and LiCl significantly induced the MMP9 transcript expression (Fig. 3B, bottom) by approximately 20-fold in both RCC cells, as well as MMP9 enzyme activity (Fig. 3C). However, inhibition of the PI3K/Akt pathway by LY294002 suppressed TNF-α–induced MMP9 expression levels (Fig. 3B, top) and its enzyme activity (Fig. 3C). LY294002 treatment also restrained TNF-α–mediated cellular migration and invasion (Fig. 3D and E, top). Upon stimulation with LiCl alone, the mobility of RCC cells were upregulated, which was the same as in response to TNF-α treatment and combination of LiCl with TNF-α treatment (Fig. 3D and E, bottom). Therefore, PI3K/Akt and GSK-3β signaling play important roles in TNF-α–stimulated MMP9 expression as well as cellular migration and invasion.

Figure 2. The changes of E-cadherin and vimentin by TNF-α were through PI3K/Akt and GSK-3β signaling in RCC. A, TNF-α stimulated the phosphorylation of Akt and GSK-3β in 786-O cells. Tumor cells were treated with TNF-α (50 ng/mL) for different time and then Western blotting with specific antibodies. B, TNF-α–stimulated EMT was through regulation of PI3K/Akt and GSK-3β. 786-O and Caki-1 cells were pretreated with LY294002 (top left and right) or LiCl (bottom left and right) for 18 hours and then stimulated with TNF-α for 4 days. C and D, TNF-α–stimulated expression of E-cadherin repressors was via regulation of PI3K/Akt and GSK-3β. 786-O and Caki-1 cells were pretreated with LY294002 (C) or LiCl (D) for 18 hours and then stimulated with TNF-α for 24 hours. Expressions of the transcriptional repressors of E-cadherin in the cells were analyzed by qRT-PCR. E, TNF-α–regulated vimentin and F-actin expression was through inactivation of GSK-3β. 786-O cells were pretreated with LiCl for 18 hours and then stimulated with TNF-α (50 ng/mL) for 4 days. Expressions of vimentin and F-actin were analyzed by immunofluorescence. Results are representative of 3 independent experiments. * P < 0.05.
active GSK-3β (S9A), or constitutively inactive GSK-3β (K85A) plasmids to test their oncogenic effects. The S9A mutant, which was unable to be phosphorylated at the Ser9 residue, was served as a constitutively active form of GSK-3β.

The K85A mutant was deficient in kinase activity and thus represented a dominant-negative form of GSK-3β (13). Overexpressions of these exogenous GSK-3β proteins in RCC cells were verified by immunoblotting using an anti-HA antibody (Fig. 4A, 5A, and Supplementary Figs. S2A and S3A).

Overexpression of S9A-GSK-3β resulted in blocking the TNF-α-induced protein expression switch between E-cadherin and vimentin (Fig. 4A and Supplementary Fig. S2A). The same results were also observed in F-actin and vimentin upon immunofluorescence staining and the cellular morphologic changes of RCC cells transfected with S9A-GSK-3β (Fig. 4B and Supplementary Fig. S2B). Moreover, the downstream targets responsible for TNF-α–induced EMT such as ZEB1, Slug, and MMP9 (Fig. 4D and Supplementary Fig. S2D), as well as the MMP9 enzyme activity (Fig. 4E and Supplementary Fig. S2E), were also inhibited by overexpression of S9A-GSK-3β. In addition, the TNF-α–induced oncogenic activities such as colony formation (Fig. 4C and Supplementary Fig. S2C), increased RCC migration, and invasion were all suppressed by S9A-GSK-3β (Fig. 4F and Supplementary Fig. S2F). Therefore, constitutive activation of GSK-3β inhibits TNF-α–induced oncogenic activities in RCC.

Overexpression of inactivated GSK-3β enhanced transformation, migration, and invasion of RCC cells

To further address the role of GSK-3β in the TNF-α–mediated tumorigenesis of RCC, we transfected RCC cells with dominant-negative K85A-GSK-3β to check its effect on various oncogenic activities. Overexpression of K85A-GSK-3β in RCC cells 786-O and Caki-1 displayed a significantly lower level of E-cadherin and higher level of vimentin (Fig. 5A and Supplementary Fig. S3A). A predominantly mesenchymal phenotype was observed in RCC.
cells transfected with K85A-GSK-3\(\beta\), accompanied by increased levels of cytoplasmic vimentin and F-actin (Fig. 5B and Supplementary Fig. S3B). In addition, RNA levels of E-cadherin repressors, ZEB1 and Slug, were increased by K85A-GSK-3\(\beta\) overexpression (Fig. 5D and Supplementary Fig. S3D). Furthermore, transduction of K85A-GSK-3\(\beta\) resulted in increased levels of MMP9 (Fig. 5D and E, and Supplementary Fig. S3D and S3E), anchorage-independent growth (Fig. 5C and Supplementary Fig. S3C) as well as enhanced mobility of RCC cells (Fig. 5F and Supplementary Fig. S3F). These effects of the K85A-GSK-3\(\beta\)–transfected RCC cells were the same as TNF-\(\alpha\)–treated cells. It was noted that TNF-\(\alpha\) and K85A-GSK-3\(\beta\) had synergistic effects on cellular mobility of RCC cells (Fig. 5F and Supplementary Fig. S3F). These data indicated that inactivation of GSK-3\(\beta\) promotes tumorigenicity of RCC by enhancing cellular transformation, migration, and invasion.

Inactivation of GSK-3\(\beta\) enhanced tumorigenicity of RCC in vivo

To investigate further the role of GSK-3\(\beta\) involvement in RCC tumorigenicity in vivo, 786-O cells transfected with vector, K85A-GSK-3\(\beta\), or S9A-GSK-3\(\beta\) plasmids were injected into nude mice. K85A-GSK-3\(\beta\) significantly promoted tumor growth (1,024 mm\(^3\)/60 d) compared with vector control pcDNA3 (609 mm\(^3\)/60 d), which is comparable with mice bearing tumors from cells treated with TNF-\(\alpha\) (Fig. 6A). In contrast, S9A-GSK-3\(\beta\) robustly attenuated tumor growth in nude mice (282 mm\(^3\)/60 d; Fig. 6A). Western blotting and qRT-PCR analysis of tumors harvested from mice showed that TNF-\(\alpha\) treatment as well as K85A-GSK-3\(\beta\) transfection of RCC stimulated Ser9 phosphorylation of GSK-3\(\beta\) protein, increased levels of vimentin protein and MMP9 mRNA, and decreased amount of E-cadherin protein in vivo (Fig. 6B).
To study whether GSK-3β inactivation and EMT exist in human RCC, we analyzed the expression of GSK-3β, E-cadherin, and vimentin in 8 pairs of normal and tumor tissues obtained from RCC patients. GSK-3β was expressed in both tumor and corresponding normal renal tissues at a similar level. However, the Ser9 phosphorylation of GSK-3β was significantly increased by 15-fold in tumor samples. Consistently, a 2-fold increase in vimentin and 3-fold decrease in E-cadherin were present in tumors (Fig. 6C and D). These results indicated that the inactivation of GSK-3β does play an important role in regulating EMT and is essential for the TNF-α–mediated tumorigenicity of RCC.

Discussion

Cancer-related inflammation assists the proliferation and survival of malignant cells, stimulates angiogenesis and metastasis, disrupts adaptive immunity, and alters response to hormones and chemotherapy (21, 22). TNF-α, a major mediator of cancer-related inflammation, can induce generation of different types of mediators, including reactive oxygen intermediates, cyclooxygenase, MMPs, and cytokines (6). RCC cells with mutated von Hippel–Lindau (VHL) and high malignant potential (2, 3) were found to produce increased low-level of TNF-α secretion (~10 pg/mL cells) (5, 17, 23). Coexpression of TNF-α and its receptors in RCC cells results in autocrine- and/or paracrine-stimulated growth (8, 21). However, the pathologic mechanism of TNF-α in RCC tumor progression remains unclear. In this study, we showed that stimulation of TNF-α promoted transformation, migration as well as invasion. But TNF-α treatment did not induce cell death in VHL-mutant (786-O and A498) and VHL-low (Caki-1) RCC cells. Besides, inhibition of PI3K

Figure 5. Inactivation of GSK-3β was involved in the transformation, migration, and invasion of RCC cells. A–C, 786-O cells were transiently transfected with empty vector (pcDNA3) or constitutively inactive GSK-3β plasmid (K85A). After 48 hours, these cells were treated with TNF-α (50 ng/mL) for 4 days or 1 hour. The expressions of target proteins were examined by Western blotting (A) and immunofluorescence (B). The number of formed colonies in soft agar was observed on day 21 (C). D and E, expressions of E-cadherin repressors and MMP9 were increased by inactivation of GSK-3β. Transfected cells were treated with TNF-α for 24 hours or 4 days. The levels of E-cadherin repressors and MMP2/9 were detected by qRT-PCR (D) and gelatin zymography (E). F, transfected cells were treated with TNF-α for 4 days and the activities of migration (top) and invasion (bottom) were analyzed by Transwell assay. Ten fields were counted per filter in each group. Results are representative of 3 independent experiments. *, P < 0.05; **, P < 0.01.
prevented the TNF-α–induced tumor progression of RCC. Inactivation of GSK-3β increased the TNF-α–induced tumor progression of RCC. The results indicated that activation of PI3K/Akt and inactivation of GSK-3β play important roles in the TNF-α–induced tumor progression of RCC.

Although TNF-α was selectively toxic to malignant cells in tissue culture studies, many of these data were generated in the presence of metabolic inhibitors such as actinomycin D, cyclohexamide, or in combination with IFN-γ (24–27). TNF-α is indeed weakly cytotoxic or cytostatic to malignant cells. Its cytotoxic potential is unmasked only in combination with metabolic inhibitors; the default cell survival and inflammatory pathways downstream of TNF-α signaling are inactivated by the metabolic inhibitors allowing apoptosis to proceed (5). Despite the fact that high-dose administration of TNF-α has been used as a cytotoxic agent, recent preclinical cancer models have provided critical evidence to support the link between chronic, low level TNF-α exposure and acquisition of promalignant phenotype. TNF-α may promote cancer transformation, proliferation, angiogenesis, invasion, and metastasis (5). Animal models provide evidence that TNF-α enhances growth and spread of syngeneic, xenogeneic, and carcinogen-induced tumors of the skin, ovary, and pancreas (28–30). TNF-α and TNF-α receptor-deficient mice have reduced susceptibility to chemically induced skin cancers and develop fewer experimental metastases (31–33). In patients with advanced cancer, TNF-α antagonists are more likely to be active in combination with other treatments. Antiangiogenic agents such as bevacizumab could be good candidates to combine with anti-TNF-α treatments. By neutralizing TNF-α, the host cells in the tumor microenvironment can be reeducated from a pro- to an antitumor phenotype, then TNF-α antagonists may contribute to immunotherapy approaches (34, 35).

Figure 6. Inactivation of GSK-3β promoted tumorigenicity in vivo and GSK-3β was inactivated in human RCC tumor tissues. A and B, 786-O cells were transiently transfected with empty vector (pcDNA3), constitutively active GSK-3β plasmid (S9A) or constitutively inactive GSK-3β plasmid (K85A). After 48 hours, transfected cells were treated with TNF-α (50 ng/ml) for 6 days. Nude/scid mice (n = 6) were then subcutaneously injected with the different transfectants and tumor size was monitored (A). After 60 days, levels of total and phospho-GSK-3β (Ser9), E-cadherin, vimentin, MMP2, and MMP9 in the tumor mass were detected by Western blotting and qRT-PCR (B). C, expressions of GSK-3β, E-cadherin, and vimentin were analyzed by Western blotting in 8 pairs of normal (N) and tumor tissues (T) obtained from patients with RCC (top). Protein levels were quantified using Fuji Multi GaugeV3.0 Image software and normalized with catenine in normal and RCC tissues (bottom). The average fold of protein expressions in RCC tissues compared with normal counterparts (*, P < 0.05). D, expressions of vimentin, E-cadherin, and phospho-GSK3β (Ser9) in human RCC were determined by immunohistochemistry. Staining of vimentin and phospho-GSK3β (Ser9) were enriched in tumor part (T; bottom). Higher E-cadherin expression was detected in adjacent normal tissues (N; top). The tissues and tumors were examined at ×400 magnification.
The PI3K/Akt pathway is constitutively activated in different types of cancers and plays a critical role in tumor progression (36, 37). Activation of PI3K/Akt regulates the function of a broad array of intracellular proteins involved in fundamental processes, including cell proliferation, cell death, cell motility/adhesion, cell transformation, and neo-vascularization (36, 37). PI3K inhibition enhances chemotherapy-induced tumor cell apoptosis in cancer cells (20). Low levels of activated Akt are observed in human RCC cells (20). Some important factors, including EGF, VEGF, and TGF-β, are involved in the tumorigenesis of human carcinoma through activation of PI3K/Akt signaling cascade (38–40). These growth factors derived from RCC may be responsible for the constitutive activation of Akt. In addition, TNF-α is also secreted by tumor-associated inflammatory cells and RCC (17). TNF-α-stimulated activation of PI3K/Akt and further enhanced tumor progression in RCC. The TNF-α-enhanced Akt phosphorylation and tumor progression were blocked by treatment with PI3K inhibitor. Therefore constitutive activation of Akt in RCC may be partially due to overexpression of TNF-α.

GSK-3β is mainly a negative regulator, keeping its target turned off under resting conditions. The dysregulation of GSK-3β is involved in tumorigenesis. A dramatic increase of the inactive form of GSK-3β (pGSK-3βSer9) was observed in squamous cell carcinomas (41), suggesting that the activity of GSK-3β was downregulated during murine skin carcinogenesis. In addition, transduction of kinase-inactive GSK-3β in adult mouse mammary glands enhanced mammary tumorigenesis (12), indicating that inactivation of GSK-3β activity promotes oncogenesis of mammary epithelial cells. We found that a high amount of GSK-3β was phosphorylated at Ser9 in human RCC clinical samples and was correlated with the expression of EMT markers. Overexpression of the inactive form of GSK-3β (K85A-GSK-3β) in RCC cells promoted transformation and mobility, resulting in accelerating tumor growth in a mouse model. Constitutive activation of GSK-3β in RCC cells transfected with the active form of GSK-3β (S9A-GSK-3β) led to blocking of the TNF-α–induced tumor progression. These observations support that GSK-3β is a tumor suppressor in the tumorigenesis of the skin, mammary, as well as kidney.

In addition to its role in normal embryonic development, EMT has been found in a variety of solid tumors and is closely correlated with tumor invasion, metastasis, and unfavorable prognosis (18, 42, 43). Common molecular markers for EMT are the increased expression of vimentin and loss of E-cadherin (18). Vimentin-transduced cancer cells exhibit increased invasiveness and tumorigenicity (44). The loss of E-cadherin is associated with poor clinical outcomes in cancer cells (42, 43). Besides participation in tumor growth, GSK-3β is also involved in neoplastic transformation and metastasis (45, 46). GSK-3β directly phosphorylates β-catenin and transcriptional repressors of E-cadherin to promote degradation of these proteins. Therefore, inhibition of GSK-3β activity is required to promote transcriptional upregulation of MMPs and EMT (46, 47). In this study, TNF-α–induced inactivation of GSK-3β to promote EMT may be directly through β-catenin and/or transcriptional repressors of E-cadherin.

TNF-α could induce activation of NF-κB in the tumor cells (4). Recent findings suggest an important role for NF-κB in tumorigenesis. NF-κB activity regulated by GSK-3β is either positive or negative depending on the cell types (11). In colon and pancreatic cancer cells, GSK-3β upregulates NF-κB activity, which induces the growth of the cancer cells. However, GSK-3β is reported to downregulate NF-κB activity in neuronal cells and astrocytes. We also found stimulation of TNF-α–induced promoter activity of NF-κB in RCC cells. However, NF-κB activation was not essential for TNF-α–induced EMT and invasion of RCC cells (48).

Advanced RCC is resistant to chemotherapy and radiotherapy. The molecular mechanism of resistance to chemotherapy in advanced human RCC has yet to be elucidated. Previous studies have reported that reactivation of GSK-3β results in different types of cancer cells sensitive to chemotherapy drugs (11, 15). Inhibition of GSK-3β activity by treatment with lithium reduces chemotherapeutic induced apoptosis (11, 15). These data suggest that constitutive activation of GSK-3β in RCC may restore drug sensitivity. In addition, we found that reactivation of GSK-3β in the TNF-α–treated RCC reduces tumor transformation as well as mobility. Taken together, inactivated GSK-3β may be a prognostic factor for the progression of RCC, and GSK-3β may be a novel target for the treatment of RCC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conceptualization and design: M.-Y. Ho, S.-J. Tang, G.-H. Sun, K.-H. Sun
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-Y. Ho, S.-J. Tang, M.-J. Chuang, G.-H. Sun, K.-H. Sun
Writing, review, and/or revision of the manuscript: M.-Y. Ho, S.-J. Tang, T.-J. Chu, G.-H. Sun, K.-H. Sun
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References


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