FoxO3a mediates transforming growth factor-β1-induced apoptosis in FaO rat hepatoma cells

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INTRODUCTION

Apoptosis, or programmed cell death, is a crucial mechanism to remove excess or damaged cells during liver development and regeneration (1,2). An imbalance between the cell-proliferation and death pathways in the liver leads to the loss of tissue homeostasis and the onset of various diseases. Indeed, insufficient apoptosis has been associated with the development and progression of hepatocellular carcinoma (3).

Transforming growth factor-β1 (TGF-β1) is an important cytokine of cell homeostasis in the liver that inhibits cell proliferation and induces cell death (4-6). TGF-β primarily shows its apoptotic effect via release of cytochrome c and by activating the apoptotic protease-activating factor 1 (Apaf-1) apoptosome complex, which stimulates the caspase cascade (7). In addition, recent evidence suggests that upregulation of Bim (Bcl-2-interacting modulator of cell death) and death-associated protein (DAP) kinase, posttranslational modification of Bcl-2-associated death protein (BAD), and production of mitochondrial reactive-oxygen species (ROS) mediate TGF-β1-dependent apoptosis by linking Smads to mitochondrial-based proapoptotic events (8-11). However, the upstream signaling mechanisms that are responsible for mediating this death process are still poorly understood.

FoxO3a is a member of the FoxO family and an important regulator of apoptosis. This work aimed to elucidate the involvement of FoxO3a in TGF-β1-induced apoptosis in FaO rat hepatoma cells. TGF-β1 caused a time-dependent activation of FoxO3a and a subsequent increase in FoxO response-element-containing luciferase reporter activity, which was Akt-sensitive. The FaO cells stably transfected with a wild type FoxO3a were more susceptible to the formation of apoptotic bodies, populations of sub-G1 apoptotic cells, and collapse of the mitochondrial-membrane potential triggered by TGF-β1. It thus appears that FoxO3a plays a crucial mediatory role in the TGF-β1 signaling pathway leading to apoptosis. [BMB reports 2008; 41(10): 728-732]

RESULTS AND DISCUSSION

Activation of FoxO3a by TGF-β1 in FaO hepatoma cells

The functional activity of the FoxO3a transcription factor is tightly controlled by post-translational modification, which includes phosphorylation (16). As a first step to assess the involvement of FoxO3a during TGF-β1-induced apoptosis, we...
examined the effect of the TGF-β1 phosphorylation of FoxO3a. Treatment of FaO cells with 5 ng/ml of TGF-β1 for varying time periods decreased the phosphorylation of FoxO3a. This effect was evident at 5 min and reached a maximum at 10 min (Fig. 1A). Conversely, TGF-β1-mediated a decrease in the FoxO3a phosphorylation level that was dose-dependently blocked by SB431542, a type I TGF-β receptor (TGF-βRI) inhibitor (Fig. 1B). Consistent with this, infecting FaO cells with adenovirus carrying ALK5 (KR), a dominant-negative form of TGF-βRI, also reversed the suppressive effect of TGF-β1 on FoxO3a phosphorylation (Fig. 1C).

Because TGF-β1 markedly decreased phosphorylation of FoxO3a, we examined changes in FoxO3a transcriptional activity in TGF-β1-treated FaO cells. TGF-β1 markedly increased expression of the FRE-Luc reporter gene, in which transcription of the luciferase gene is controlled by the FoxO response element, in a dose- and time-dependent manner (Fig. 2A, B). SB431542 significantly abolished TGF-β1-induced FRE-Luc activation in FaO cells, whereas LY294002, a specific inhibitor of PI 3-kinase, had a synergic effect on FRE-Luc activation in the same cells (Fig. 2C). Consistent with this result, TGF-β1-induced FRE-Luc activation was markedly enhanced by cotransfection with pHMV6-Akt (KM) coding for Akt (KM), a kinase-inactive form of Akt, whereas it was dramatically suppressed by cotransfection with pHMV6-Akt (myristylated-ΔPH) coding for Akt (myristylated-ΔPH), a constitutively active form of Akt (Fig. 2D). These findings suggest that FoxO3a specifically mediates TGF-β1 signaling in FaO hepatoma cells.

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To further investigate the role of FoxO3a in the TGF-β1 signaling of apoptosis, a stable cell line was generated that expresses an HA-tagged wild type FoxO3a. FaO cells stably transfected with pcDNA3-HA-FoxO3a appeared to be more susceptible to TGF-β1-induced apoptosis, as typical apoptotic phenomena such as cell shrinkage, loss of cell-to-cell contact, and membrane blebbing (Fig. 3A) gave rise to an increased population of sub-G1 phase cells compared to control cells (Fig. 3B). In addition, the presence of TGF-β1 resulted in an increased loss of mitochondrial membrane potential (ΔΨm) in the FoxO3a-expressing FaO cells (Fig. 3C). In contrast, FaO cells transfected with siRNA specific for FoxO3a were less susceptible to TGF-β1-induced activation of caspase (Fig. 3D), indicating that FoxO3a is required for TGF-β1-induced apoptosis of hepatoma cells.

FoxO3a is a member of the FoxO subfamily of Forkhead transcription factors and is well known as an important regulator of apoptosis. However, its role in TGF-β1 signaling of apoptosis remains unknown. In the present study, TGF-β1 decreased phosphorylation of FoxO3a and induced FoxO3a-specific phosphorylation of FoxO3a.
mediated transcriptional activity, and this effect was reversed by a TGF-β type I receptor inhibitor. In addition, the TGF-β1-induced increase in FoxO3a activity was abrogated by myristylated-APH, a constitutively active form of Akt, further confirming that FoxO3a is a specific player in TGF-β1-mediated signaling. FAO cells that stably overexpressed FoxO3a were more sensitive to TGF-β1-induced apoptosis compared with control cells. In contrast, the TGF-β1-induced increase of caspase activity was significantly abolished by silencing FoxO3a with two independently isolated sets, and the results were averaged.

**Immunoblotting analysis**
Western blotting was performed as described previously (23) using antiphospho-FoxO3a (Cell Signaling Technology, Beverly, MA, USA), anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma, St. Louis, MO) antibodies. Immunoblot signals were developed using SuperSignal Ultra chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL).

**Flow cytometric analysis**
For the flow-cytometric assay (24), hepatoma cells were grown with the present results, these findings propose that repressing the PI 3-kinase/Akt pathway by phosphatase may be a key mechanism for the activation of FoxO3a in the TGF-β1 signaling of apoptosis. Further studies elucidating the linkage between TGF-β1 and FoxO3a will probably be pivotal to the complete understanding of TGF-β1-evoked intracellular signaling of apoptosis.

**MATERIALS AND METHODS**

**Reagents**
The recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, USA). LY294002 was purchased from Calbiochem (Merck Biosciences, USA). The N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) and dihydrorhodamine 123 were purchased from Enzyme Systems Products (Dublin, CA) and Molecular Probes (Eugene, OR).

**Cell culture and generation of stable cell lines**
FAO rat hepatoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and streptomycin (100 μg/ml) at 37°C under a humidified 95/5% (v/v) mixture of air and CO2. For the stable expression of FoxO3a, FAO cells were cotransfected with pECE-HA-FoxO3a and the pCDNA3 expression plasmid using FuGENE 6 (Roche, Mannheim, Germany). Stably transfected clones were selected with 0.5 mg/ml neomycin (Invitrogen, Carlsbad, CA). After 2 weeks of selection, neomycin-resistant colonies were analyzed for HA-FoxO3a expression by immunoblotting the cell lysates with anti-HA antibody.

**DNA transfection and luciferase assay**
The FAO cells were transfected using FuGENE 6 (Roche, Mannheim, Germany). To control for variation in transfection efficiency, all clones were cotransfected with 0.2 μg of CMV-β-GAL, a eukaryotic expression vector in which the Escherichia coli β-galactosidase (Lac Z) structural gene is under the transcriptional control of the CMV promoter. Luciferase reporter activity was assessed on a luminometer with a luciferase assay system (Promega, Madison, WI) according to the manufacturer’s protocol. Transfection experiments were performed in triplicate with two independently isolated sets, and the results were averaged.

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in six-well plates, incubated for 24 h at 37°C, and then treated with TGF-β1. After 24 h, the cells were harvested and washed twice with PBS (pH 7.4). After fixing in 80% ethanol for 30 min, the cells were washed twice and resuspended in PBS (pH 7.4) containing 0.1% Triton X-100 and 5 μg/ml propidium iodide (PI), and then analyzed by a FACScan cytometer (Program CellQuest, BD Biosciences).

### Caspase-3 assay
Caspase-3 activity in cytosolic extracts was determined with a spectrophotometric assay, as described previously (25). Briefly, the peptide substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) was added to the cell lysates in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol) and incubated at 37°C. The cleavage of the substrate was monitored at 405 nm.

### Assessment of mitochondrial transmembrane potential
Changes in mitochondrial membrane potential were determined by staining the cells with the fluorescence probe dihydrorhodamine 123 (Molecular Probes, Eugene, OR). The cells were incubated in phosphate-buffered saline (PBS) containing 10 μM dihydrorhodamine 123 (Rh-123) for 30 min at 37°C in the dark and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The fluorescence was excited with an Argon laser (excitation wavelength, 488 nm) and analyzed in FL-1 (wavelength, 520 nm; photomultiplier tube [PMT] voltage, 437 V). At least 2 x 10^5 events were acquired in list mode and analyzed with CELLQuest software (Becton Dickinson, San Jose, CA).

### Statistical analysis
All data are expressed as mean ± SD and are representative of three or more independent experiments. Statistical analyses were assessed by Student’s t test for paired data. Results were considered significant at P < 0.05.

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### REFERENCES
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