Transforming growth factor-β1-induced Smad signaling, cell-cycle arrest and apoptosis in hepatoma cells

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Transforming growth factor-β1 (TGFβ) is involved in the regulation of liver cell proliferation and apoptosis, and escape of hepatoma cells from the growth restraining signals of TGFβ has been suggested to contribute to tumor development. TGFβ modulates gene transcription by receptor-mediated activation of Smad proteins which act as transcription factors. TGFβ-mediated primary signaling responses as well as effects on the cell cycle and apoptosis were investigated in the human hepatoblastoma line HepG2, the rat hepatoma line FTO-2B and the mouse hepatoma line 55.1c. Activation of a Smad (Sma and Mad homolog) response-element-driven luciferase reporter by TGFβ was very similar in all three cell lines, indicating functionality of the primary TGFβ signaling pathway. Moreover, TGFβ-inducible early gene was transiently activated by TGFβ in all cell lines as shown by RT–PCR. HepG2 cells, however, were completely resistant to TGFβ-induced growth arrest and apoptosis and 55.1c cells were only slightly susceptible to TGFβ-induced apoptosis. By contrast, treatment of FTO-2B cells with TGFβ led to a partial G0/G1 arrest and a strong induction of apoptosis. TGFβ-induced apoptosis of FTO-2B cells was inhibited by dexamethasone, insulin, phenobarbital and dieldrin. Of these agents, only insulin led to a significant reduction of TGFβ-stimulated Smad-reporter activity, suggesting that the other compounds interfere with TGFβ-induced apoptosis downstream of Smad-mediated primary transcriptional responses at a level that may be constitutively altered in apoptosis-resistant hepatoma cell lines.

Introduction

Transforming growth factor-β1 (TGFβ) is the prototype of a family of cytokines that are involved in regulation of various cellular responses including cell growth, differentiation and apoptosis (1,2). TGFβ is synthesized and secreted as a latent protein, which is known to interfere with TGFβ signaling, cell-cycle arrest and apoptosis. TGFβ-mediated apoptosis of FTO-2B cells is transiently activ-

Abbreviations: Cdk, cyclin-dependent kinase; EGR, early growth response; Erk1/2, extracellular signal-regulated kinases 1/2; M6P/IGF2, mannose-6-phosphate/insulin-like growth factor-II; PBS, phosphate buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; SBE, Smad binding element; Smad, Sma and Mad homolog; TGFβ, transforming growth factor-β1; TIEG, TGFβ-inducible early gene.

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medium (DMEM), HepG2 cells (ATCC, Rockville, USA) in alpha minimum essential medium and 55.1c cells (24) in DMEM supplemented with 0.2 g/l L-arginin in a humidified incubator (5% CO_2, 37°C). All media contained 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin). Culture media and essential serum were obtained from Gibco (Eggenstein, Germany) antibodies from Biochrom (Berlin, Germany) and culture dishes from Becton Dickinson (Heidelberg, Germany).

Apoptosis and cell cycle analyses

FTO-2B, HepG2 and 55.1c cells were plated on 3.5 cm culture dishes (4–6×10^5 cells per dish). After 24 h (FTO-2B and 55.1c) or 48 h (HepG2), cells were washed with phosphate buffered saline (PBS), incubated in fresh medium and treated with TGFβ and/or apoptosis inhibitors. TGFβ (R&D Systems, Wiesbaden, Germany) was used at a concentration of 5 ng/ml medium (1 µg/ml stock in 4 mM HCl/1% BSA). Concentrations of anti-apoptotic agents were as follows (solvents are given in parentheses): 2 mM phenobarbital (PBS), 25 µM dieldrin (DMSO), 10–8 M dexamethasone (PBS/10% ethanol) and 10–7 M insulin (PBS/1% acetic acid). Anti-apoptotic compounds were prepared as 1000× stocks, except for phenobarbital, where a 100× stock was used. Phenobarbital was obtained from Geyer (Renningen, Germany) and dieldrin, dexamethasone, and insulin from Sigma (München, Germany). TGFβ/solvent controls were incubated with TGFβ and the solvent of the respective apoptosis inhibitor. In some experiments cells were irradiated with UV light (200 J/m^2) using a DNA-crosslinker (Bioterrnt, Göttingen, Germany).

Apoptosis was quantitated by determination of caspase-3-like activity as described previously (19) using N-acetyl-Asp-Glu-Val-Asp-nitroanilide as a substrate. Flow cytometric analyses were performed essentially according to Nicoli et al. (25). In brief, cells were detached from the culture dishes by Trypsin/EDTA treatment, washed with PBS and incubated with propidium iodide [50 µg/ml in 0.1% (w/v) sodium citrate/0.1% (v/v) Triton X-100] for 5 min. Propidium iodide fluorescence was measured on the FACS Calibur (Becton Dickinson) and cell cycle distribution, as well as subdiploid (apoptotic) nuclei, were quantitated using CellQuest 5.0.1.f and ModFit LT V2.0 software.

Transfection experiments and reporter assays

FTO-2B, HepG2 and 55.1c cells were plated on 24-well plates (1–2×10^5 cells/well) and transfected with 1–2 µg/well of the plasmid (kindly provided by Dr W.Kruijer, Haren, The Netherlands) using Unifectin-M (kindly provided by Dr A.Surovoy, Tübingen, Germany). This plasmid contains four copies of the Smad binding element (SBE) driving the Firefly luciferase reporter gene (26). Cells were co-transfected with 5–10 ng/well of the Renilla luciferase reporter plasmid pRL-CMV (Promega, Mannheim, Germany) which was used for normalization of transfection efficiencies. After 16 h of transfection, cells were washed with PBS, incubated in fresh medium and treated for 8 h (FTO-2B) or 24 h (HepG2 and 55.1c) with TGFβ and/or apoptosis inhibitors at the concentrations described above. Each experiment contained six separate transfections per treatment and was repeated at least three times. Cells were lysed directly on the plate and luciferase activities were determined on a Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany). Chemiluminescence signals were monitored using a CCD camera system (Raytest, Straubenhardt, Germany) and quantitated by image analysis (TINA V2.09g, Raytest).

**Fig. 1.** Activation of Smad signaling by TGFβ in FTO-2B, HepG2 and 55.1c cells. Cells co-transfected with pGL3ti-(SBE)_4 and pRL-CMV plasmids were incubated with 5 ng/ml TGFβ or solvent for 8 h (FTO-2B) or 24 h (HepG2 and 55.1c). Luciferase activities were determined by the dual-luciferase reporter system as described in Materials and methods. Fold induction of reporter activity by TGFβ (means ± SD) from three to five experiments, each consisting of six independent transfections per treatment, is shown (P < 0.05, **P < 0.01; Student’s t-test).

**Results**

Many of the effects of TGFβ are mediated through activation of Smad proteins which, in turn, lead to transcriptional activation of responsive genes (6). To analyze this primary signaling step in hepatoma cells, FTO-2B, HepG2 and 55.1c cells were transiently transfected with the TGFβ-responsive luciferase reporter construct pGL3ti-(SBE)_4 containing four copies of the SBE. As shown in Figure 1, treatment of FTO-2B cells with TGFβ for 8 h led to a significant activation of the reporter gene (2.2-fold increase over untreated controls). If cells were incubated with TGFβ for up to 16 h, only a slight further increase in reporter activity was obtained (2.4-fold at 12 h and 2.7-fold at 16 h); analyses at later times were not possible, since a high proportion of cells were triggered into apoptosis (see below). In 55.1c and HepG2 cells, TGFβ led to an ~2–3-fold stimulation of reporter activity over untreated controls after 24 h of treatment (Figure 1). These results indicate that all three cell lines contain a functional TGFβ receptor system and display similar primary TGFβ3 responses.

To study the biological consequences of Smad activation, the effects of TGFβ on cell cycle and apoptosis were analyzed in FTO-2B and HepG2 cells. While TGFβ led to a slight, but significant cell-cycle arrest in FTO-2B cells, as shown by a reduction in the percentage of cells in S-phase and a concomitant rise of cells in G0/G1, no such effect was seen in HepG2 cells (Figure 2). Differential responses of the
two cell lines were also obtained with respect to TGFβ-induced apoptosis, which was analyzed by determination of caspase-3-like activity (Figure 3). Treatment of FTO-2B cells with TGFβ for 24 h led to an ~16-fold increase in caspase-3-like activity over untreated controls, indicating a strong apoptotic response in this cell line. Similar results were obtained by cytofluorometric analysis of subdiploid apoptotic nuclei (see Figure 6). HepG2 cells, in contrast, did not show any increase in caspase-3-like activity upon TGFβ treatment for 24 h (Figure 3). Analyses for up to 48 h after TGFβ application also revealed no signs of apoptosis (data not shown), demonstrating that HepG2 cells are resistant to TGFβ-induced apoptosis, which is in accordance with findings of others (20, 21). HepG2 cells, however, do not show a general defect in apoptotic response, since the use of UV irradiation as a trigger of apoptosis led to a sharp increase in caspase-3-like activity (Figure 3) and appearance of apoptotic cells (data not shown). Similarly, 55.1c cells are almost completely resistant to TGFβ-induced apoptosis (Figure 3), but show a strong apoptotic response after UV irradiation (27).

The difference between FTO-2B, HepG2 and 55.1c cells in their response to TGFβ could be due to differences in the expression of cell-cycle and apoptosis-associated proteins. In various cell lines, TGFβ-induced cell-cycle arrest has been shown to be associated with transcriptional activation of inhibitors of Cdns (7). Analysis of two representative candidates, i.e. p21<sup>Cip1/Waf1</sup> and p15<sup> Ink4B</sup>, Bcl-2, Bcl-x<sub>L</sub> and Bax, respectively. An anti-Actin antibody was used to control for equal loading of lanes. Total protein (10–50 µg) was used for analysis of Bcl-2, Bcl-x<sub>L</sub>, Bax, and p21<sup>Cip1/Waf1</sup>, while 250 µg was used for analysis of p15<sup> Ink4B</sup>. Chemiluminescence signals were monitored using a CCD camera system. The insert shows a longer exposure of the Bcl-2 blot (HepG2 cells). Quantitation of chemiluminescence signals by image analysis revealed a moderate induction (~2-fold at maximum) of p21<sup>Cip1/Waf1</sup> by TGFβ in all three cell lines. In addition, an ~50% decrease in Bcl-x<sub>L</sub> and an ~2-fold increase in Bcl-2 levels by TGFβ was observed in 55.1c cells. Expression of the other proteins was not significantly affected by TGFβ treatment.

Induction of apoptosis by TGFβ may be mediated by transcriptional activation of the TGFβ-inducible early gene (TIEG). We therefore comparatively analyzed the effects of TGFβ on TIEG1 expression by RT-PCR in FTO-2B, HepG2 and 55.1c cells. Since human TIEG1 is transcribed from the same gene as early growth response α (EGRα) (28), one of the primers used for PCR (TIEG1-F) was designed for exon 1a which is not shared by EGRα and is therefore specific for TIEG1. Alternative transcripts have also been reported for mouse TIEG (29). As shown in Figure 5, TGFβ led to a transient induction of TIEG1 in all three cell lines with a maximum at 1 h of TGFβ treatment. The level of induction in the apoptosis-resistant HepG2 cell line was comparable with that observed in the apoptosis sensitive FTO-2B cells, indicating that TIEG1 is not responsible for the differences in apoptotic response of the hepatoma cell lines tested.

We have previously shown that apoptosis of FTO-2B cells can be inhibited by a variety of endogenous and exogenous compounds, such as dexamethasone, insulin, phenobarbital and dieldrin (19). Confirming our previous observations, TGFβ-induced apoptosis was reduced by 50–80% when FTO-2B cells were treated simultaneously with TGFβ and either of these inhibitors (Figure 6). Since suppression of apoptosis by the above agents could result from an interference with...
Inhibition of TGFβ-induced apoptosis in FTO-2B cells. Cells were incubated for 24 h with 5 ng/ml TGFβ together with either $10^{-8}$ M dexamethasone (dexa), $10^{-7}$ M insulin, 2 mM phenobarbital (pheno), 25 µM dieldrin or the solvents of the anti-apoptotic compounds. Cells not treated with TGFβ (–TGFβ) received 4 mM HCl/1% BSA instead. Apoptosis was quantitated by determination of caspase-3-like activity (left) and flow cytometric analysis of subdiploid (apoptotic) nuclei (right). Values for each treatment group are expressed as a percentage of the respective TGFβ/solvent control. Means ± SD from three to four independent experiments are shown. Significant inhibition of apoptosis is indicated (*$P < 0.05$, **$P < 0.01$; Student’s t-test).

Effects of anti-apoptotic agents on activation of Smad signaling by TGFβ. FTO-2B cells were co-transfected with pGL3ti-(SBE)$_4$ and pRL-CMV plasmids and incubated with 5 ng/ml TGFβ alone or together with either dexamethasone (dexa), insulin, phenobarbital (pheno) or dieldrin at concentrations given in the legend to Figure 6. After 8 h of treatment, luciferase activities were determined as described in Materials and methods and expressed as a percentage of TGFβ controls. Means ± SD from three experiments, each consisting of six independent transfections per treatment, are shown. *Significant inhibition of TGFβ-induced reporter activity ($P < 0.02$; Student’s t-test).

TGFβ/Smad signaling, the effects of dexamethasone, insulin, phenobarbital and dieldrin on the activity of the pGL3ti-(SBE)$_4$ luciferase reporter were investigated. Reporter activity was determined in transiently transfected FTO-2B cells after simultaneous amplification of TIEG1 and β-actin by PCR. PCR products were separated on 10% polyacrylamide gels, stained with ethidium bromide, photographed and signal intensities were quantitated by image analysis. Maximal induction by TGFβ of β-actin-normalized TIEG1 signals is indicated.

TGFβ is an important physiological mediator of cell homeostasis in liver and escape of tumor cells from the growth restraining signals of TGFβ has been suggested to contribute to liver tumor development. While proliferation of normal hepatocytes is inhibited by TGFβ, several studies demonstrate that cells derived from hepatocellular tumors and even preneoplastic hepatocytes are resistant to the growth inhibitory effects of TGFβ (e.g. 22,23,30–32). Similarly, resistance of hepatoma cells to TGFβ-induced apoptosis has been reported (20,21). Alterations in function and expression of TGFβ-associated receptors have been shown to contribute to the aberrant TGFβ-response of hepatoma cells (9); however, comparatively little is known about qualitative or quantitative alterations in downstream effectors within the TGFβ pathway.

To gain an insight into the role of Smad proteins for regulation of cell cycling and apoptosis in hepatoma cells, we were interested in two questions: (i) do cell lines either responsive or refractory to TGFβ-induced growth arrest and apoptosis show differences in Smad-mediated transcriptional responses to TGFβ, and (ii) how is the primary TGFβ signaling
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pathway affected by compounds known to inhibit TGFB-induced apoptosis of hepatoma cells. Our results demonstrate that FTO-2B cells are responsive to TGFB-induced cell-cycle arrest and apoptosis, whereas 55.1c cells are largely resistant and HepG2 cells are completely resistant. Resistance of HepG2 cells, which has also been observed in previous studies (20–23), cannot be due to defects in TGFB-associated receptor systems, since they are known to possess TGFB type I and II receptors (33) and respond to TGFB with functional Smad signaling (26,34,35). We were therefore interested to know whether the extent of transcriptional responses to TGFB in HepG2 and 55.1c cells might be lower than in FTO-2B cells. Our results show that stimulation of a Smad-dependent reporter gene by TGFB was very similar in all three cell lines, indicating that the observed differential response to cell-cycle arrest and apoptosis cannot be attributed to differences in the primary TGFB signaling cascade. Similarly, no differences in induction of TIEG1, which has been implicated to be involved in induction of hepatoma cell apoptosis by TGFB (36), were observed. These observations suggest that HepG2 and 55.1c cells may have yet unknown defects in pathways downstream of Smad and TIEG1 proteins that are involved in regulation of cell growth and apoptosis.

It was shown recently that HepG2 cells contain high levels of Bcl-2 when compared with other human hepatoma cells (21), which led the authors to suggest that this anti-apoptotic protein may be responsible for the observed lack of apoptotic response in HepG2 cells. The comparison of expression levels of Bcl-2 family proteins in cells of the three lines of the present study, which differ strongly with respect to their susceptibility to TGFB-induced apoptosis, yielded no uniform results. Clearly, Bcl-2 is not generally associated with resistance of hepatoma cells to apoptosis since the level of expression of this anti-apoptotic protein was much lower in TGFB-resistant HepG2 cells when compared with TGFB-sensitive FTO-2B cells. An inverse expression pattern was found with respect to Bcl-xL, which may also mediate protection from apoptosis. Finally, the levels of Bax, which has been suggested to play a role in induction of liver cell apoptosis by TGFB (37,38), were similar in all three lines and were not affected by TGFB, indicating that the observed differences in susceptibility to TGFB-induced apoptosis are not directly related to expression of this pro-apoptotic protein.

TGFB-induced apoptosis of FTO-2B cells can be inhibited by a variety of liver tumor promoters, such as phenobarbital or dieldrin, and hormonally active compounds, such as insulin or dexamethasone. Although our previous results suggested that inhibition of apoptosis by these compounds occurs at a presumably late stage of apoptosis, but upstream of activation of caspase-3 or related proteases, we could not rule out the possibility of interference with early TGFB signaling events (19). Our present results demonstrate that phenobarbital, dieldrin and dexamethasone do not interfere with TGFB-induced Smad activation, while insulin led to an ~50% reduction of Smad-mediated transcriptional activation of a luciferase reporter. Insulin is known to activate various kinases, including extracellular signal-regulated kinases 1/2 (ERK1/2) (39), an effect which is also seen in FTO-2B cells (19). Since ERK1/2 have been shown to interfere with activation of Smad proteins (40,41), insulin-mediated inhibition of the TGFB/Smad signal transduction pathway may be due to activation of these kinases. Clearly, however, this mechanism cannot account for the other apoptosis inhibitors studied, which do not activate ERK1/2 (19) and failed to suppress Smad-mediated transcriptional responses in FTO-2B cells (this paper). This evidence indicates that TGFB-induced apoptosis can be abrogated in the presence of active Smad signaling and suggests that phenobarbital, dieldrin and dexamethasone interfere with the apoptotic program at a level downstream of TGFB/Smad signaling. The nature of this point of interference is unknown but does not appear to be related to cell-cycle control since cell-cycle distribution of FTO-2B cells was not significantly affected by the above mentioned agents (data not shown); only dexamethasone led to a partial arrest at the G1/S transition (see also 42). A differential screen for genes activated in FTO-2B, HepG2 and 55.1c cells in response to TGFB and the anti-apoptotic agents may clarify whether the suppression of apoptosis of FTO-2B cells by anti-apoptotic agents and the constitutive resistance of HepG2 and 55.1c cells to apoptosis have a common basis.

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References


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