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Induction of p53 and Bax during TGF-β1 Initiated Apoptosis in Rat Liver Epithelial Cells

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The relationship between transforming growth factor-β1 (TGF-β1) induced growth arrest and apoptosis in rat liver derived epithelial (RLE) cells was analyzed. TGF-β1 treatment of RLE cells induced both cell cycle arrest and apoptosis. However, pretreatment of the cells with either dexamethasone or cyclohexamide suppressed TGF-β1 induced apoptosis, without preventing the cell cycle arrest. Both p53 and Bax were subsequently shown to be overexpressed during the TGF-β1 induced apoptosis. Furthermore, it was revealed that cycloheximide suppressed expression of both p53 and Bax. In contrast, dexamethasone treatment prevented Bax expression alone. Treatment of RLE cells with several growth factors either alone or in combination was ineffective in counteracting TGF-β1 induced apoptosis. In additon, we show that TGF-α also induced both p53 and Bax expressions and augmented TGF-β-induced apoptosis. Thus, p53 and Bax are likely to be key factors in TGF-β1 induced apoptosis in RLE cells. **© 1998 Academic Press**

Establishment of non-parenchymal epithelial cell lines from the rat liver by utilizing culture conditions that exclude differentiated hepatocytes has been accomplished by several investigators (1). These rat liver epithelial (RLE) cells are propagatable and proliferate in long-term cultures in contrast to the limited proliferative life spans of hepatocytes and bile duct cells in vitro (1,2). The phenotypic characteristics of RLE cells have been shown to resemble those of "oval cells" which are considered as a close progeny of hepatic stem cells (1). In addition, RLE cells can differentiate into hepatocytes and bile duct cells when transplanted into the rat liver (3). Normal RLE cells are very sensitive to the growth inhibitory effects of transforming growth factor-β1 (TGF-β1) whereas most of the transformed RLE cells are resistant to the growth inhibition by TGF-β1 suggesting that disruption of TGF-β1 signaling plays a role in transformation of RLE cells (4,5).

TGF-β1 can, in addition to exerting a powerful growth inhibitory effect on a variety of epithelial cells, also induce apoptosis in these cells (6). In contrast to the well defined mechanism of growth inhibition by TGF-β1 (7) the characterization of the apoptotic effect is less advanced. The involvement of reactive oxygen was recently reported in TGF-β1 induced apoptosis of primary rat fetal hepatocytes (8). However, TGF-β1 induced apoptosis in hepatoma cell lines appears not to involve reactive oxygen species (9). These results indicate that the mechanism of TGF-β1-induced apoptosis may differ between normal and transformed hepatocytes.

The aim of this study was to use the RLE cells to characterize the molecular cascade involved in apoptosis initiated by TGF-β1 in order to provide insight into the involvement of the TGF-β signaling pathway in the regulation of hepatic stem cell biology. The present results show that TGF-β1 induced growth inhibition and apoptosis in RLE cells are independently controlled, and that p53 and Bax constitute the central components of the apoptotic pathway.

MATERIALS AND METHODS

Cell culture. The RLE cell line and the transformed variant, C4T, have been extensively characterized (5,6,19,20). They were maintained in Ham's modified F-12 medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) and 50 Tg/ml gentamicin at 37°C in 95%-5% air-CO_2. Two days later, the media were changed and the cells were subjected to various treatment regimens as indicated. The growth inhibitory and apoptotic effects of TGF-β1 (1 ng/ml, R&D Systems, Minneapolis, MN) on the RLE cells were analyzed under various conditions. Also, the impact of treating (100 ng/ml) the cells with TGF-α, HGF, SCF, IGF-1, IGF-2 and bFGF (all of which were purchased from R&D Systems), either individually or in combination on modification of the effect of TGF-β1 were also examined. Dexamethasone (Dex) and Cycloheximide (CHX) were purchased from Sigma (St. Louis, MO). The cells were treated with Dex and CHX at 40 ng/ml and 0.5 μg/ml, respectively. The protease

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inhibitor 3,4-dichloroisocoumarin (DCI) was purchased from Boehringer Mannheim (Indianapolis, IN).

FACS analysis. The RLE cells were, following the various treatment protocols, trypsinized and fixed with 75% ethanol at −20°C for at least 30 min. After centrifugation at 140 g for 5 min at 4°C, pellets were suspended in 1 ml of 0.5% Triton-X100 and incubated at room temperature for 5 min. The permeabilized cells were then stained with propidium iodide (PI) solution (50 mg/ml) containing 0.5% (w/v) RNase A. After 10 min incubation at room temperature, DNA content of samples was analyzed by FACSort flow cytometer with argon laser tuned to the 488 nm line for excitation (Becton-Dickinson, San Jose, CA).

Western blot analysis. Cells were lysed on ice with 200 μl SDS sample-buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 5% β-mercaptoethanol. The lysate was kept on ice for 10 min, followed by centrifugation at 14000 g for 15 min at 4°C to collect the supernatant. Protein concentration was determined by using Bio-Rad protein Assay Kits (Bio-Rad Laboratories, Hercules, CA). Aliquots (40 μg) of samples were separated on 10% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose filters. In order to confirm if equal amounts of whole proteins between samples were transblotted, Ponceau staining (0.1%; Sigma, St. Louis, MO) was performed. The target proteins were visualized by using primary antibodies (antibodies for p53, and Bax, Bcl-x, Bcl-2 were obtained from Pharmingen, San Diego, and Santa Cruz Biotechnology, Inc., Santa Cruz, CA, respectively), the secondary peroxidase-coupled IgG antibody (Pierce Inc., Rockford, IL) and an enhanced chemiluminescence kit (Amersham Life Sciences Inc., Arlington Heights, IL) were also used.

Nuclear extracts and electrophoretic mobility of shift assays. Nuclear extracts were prepared (10) and gel shift assays were performed as follows: Oligonucleotides for AP-1 and NF-kB and all antibodies related to AP-1 components (jun/fos) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nuclear protein extracts (5 μg) and antibodies (1 mg) were added in a standard buffer (10 mM Tris, pH 7.5; 50 mM NaCl; 1 mM MgCl2; 20% glycerol) and pre-incubated at room temperature for 15 min. The ^{32}P-endlabelled oligonucleotides (1×10^5 cpm) and 2 μg of poly(dI-dC) were then added and incubated at room temperature for an additional 15 min. DNA binding complexes were resolved by gel electrophoresis on 5% polyacrylamide/1xTBE. The gels were dried and exposed to Kodak (Rochester, NY) X-AR film at −80°C for approximately 12 h.

Antisense oligonucleotide treatment. Phosphothionate-modified antisense and sense oligonucleotides corresponding to codon 1-6 of rat *junD* and *fra2* were synthesized and purified by OPC column (Bioserve Biotechnology Inc., Laurel, MD). The sequences of the antisense and sense oligonucleotides were as follows: junD antisense: ATAGAAGGGCGTTTCCAT; junD sense: ATGGAAACGCCCTTCTAT; fra-2 antisense: GGGATAATCCTGGTACAT; fra-2 sense: ATAGAAGGGCGTTTCCAT.

Cells were grown for two days in Ham's F-12 containing 10% FBS. After washing twice with DPBS, cells were cultured for 24 h with either antisense or sense oligonucleotides at concentrations of 80 μg/ml in FBS-free medium containing 1 ng/ml of TGF-β1.

RESULTS AND DISCUSSION

TGF-β1 induces cell cycle arrest. As previously shown cell cycle arrest was completed 24 h after treatment, and inhibition of DNA synthesis and the time course of TGF-β1 induced growth arrest was not affected by addition of TGF-α, HGF, SCF, IGF-1, IGF-2 and aFGF (data not shown). All of the tested growth factors are associated with growth promoting effect during liver regeneration (11).

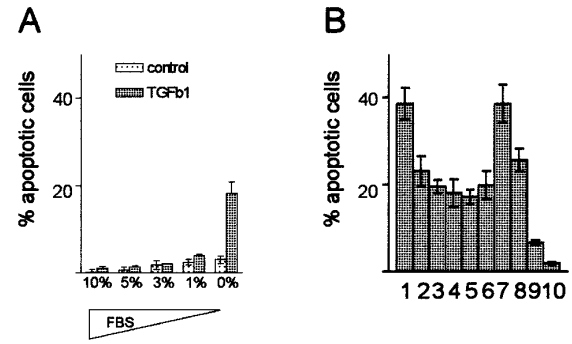

FIG. 1. Panel **A:** Effect of FBS concentration on spontaneous and TGF-β1 induced apoptosis in RLE cells. Panel **B:** Alteration of TGF-β1 induced apoptosis in RLE cells by treatment with growth factors, dexamethasone (Dex) and cycloheximide (CHX). The cells were incubated with 100 ng/ml of the growth factors in combination with TGF-β1 (1 ng/ml) for 48 h. The treatment period with Dex and CHX, 40 ng/ml and 0.5 Tg/ml, respectively, was also 48 h. Bars number **1** to **10** represent effects of the following factors on TGF-β1 induced apoptosis in RLE cells: **1.** TGF-α; **2.** HGF; **3.** SCF; **4.** bFGF; **5.** IGF-1; **6.** IGF-2; **7.** Combination of 1-6; **8.** Combination of 2-6; **9.** Dex; **10.** CHX.

TGF-β1 induced apoptosis in RLE cells. In order to maximize the apoptotic effects of TGF-β1 the RLE cells were treated under serum free conditions, resulting in 18.5% of the cell undergoing apoptosis in 48 h (Fig. 1A). Treatment with a number of growth factors associated with liver regeneration failed to protect against TGF-β1 induced apoptosis of the RLE cells (22; Fig. 1B). Among these growth factors TGF-α was capable of inducing apoptosis in the RLE cells and had in combination with TGF-β1 additive apoptotic effect (Fig. 1B). We further tested the possible protection of the glucocorticoid analog Dex, because it was reported to prevent apoptosis in mammary involution, a process in which TGF-β1 may play a causative role (12). Treatment with Dex did not affect TGF-β1 induced cell cycle arrest of RLE cells (data not shown), but effectively suppressed the apoptosis (Fig. 1B).

TGF-β1 induces p53 and Bax. In general, apoptosis is thought to be regulated by the balance between agonist and antagonist affecting the processes. The observation that TGF-β1 induced apoptosis was abolished by addition of Dex suggested at least two possible mechanisms: (1) that the treatment of the cells with Dex suppressed a death agonist(s) induced by TGF-β1, or (2) induced a death antagonist(s) which might be suppressed by TGF-β1. In an attempt to differentiate between these two possible mechanisms, the RLE cells were treated with the protein synthesis inhibitor CHX in combination with TGF-β1. The result showed that under these conditions the TGF-β1 induced apoptosis was completely abolished (Fig. 1B), suggesting that the apoptosis was dependent on production of a death agonist(s), rather than on suppression of a death antagonist(s).

Since p53 is an essential death agonist as well as an inducer of cell cycle arrest, both of which are induced in response to a wide variety of stimuli (13), we examined its expression following TGF-β1 treatment. The expression of p53 was very low in control RLE cells grown in 10% FBS, but increased in a time-dependent manner after TGF-β1 administration (data not shown). This observation suggested a possible involvement of p53 in TGF-β1 induced cell cycle arrest. However, p53 expression increased to a much greater extent following TGF-β1 treatment of RLE cells in the absence of FBS and this increase correlated with the induction of apoptosis (Fig. 2A), suggesting a role of p53 in the apoptotic process. In an attempt to further clarify the role of p53 in the TGF-J1 induced apoptosis, we examined another death agonist, Bax, which is transcriptionally upregulated by p53 (14). Bax expression increased in a time dependent manner in parallel with p53 induction (Fig. 2A), suggesting that p53 and Bax may be key factors in TGF-β1 induced apoptosis. Moreover, both p53 and Bax were induced during TGF-α initiated apoptosis which was not related to cycle arrest further supporting the role of these death agonists in the apoptosis (Fig. 2B). Also, Bcl-xL, an antagonist against apoptosis was barely detected under conditions of Dex and CHX (data not shown) abrogated apoptosis. We were unable to detect Bcl-2 expression in the RLE cells.

TGF-β1 induced apoptosis is suppressed via down-regulation of p53 and/or Bax. CHX treatment effectively reduced levels of both Bax and p53 proteins (Fig. 2B), resulting in the abrogation of apoptosis (Fig. 1B). However, Dex treatment selectively reduced Bax expression, without significantly suppressing p53 protein expression (Fig. 2A), suggesting that Dex negatively controls Bax, independent of p53. Since the suppression of Bax expression was the common event in the abrogation of apoptosis by both CHX and Dex-intervened apoptosis, Bax induction appeared to be the critical event in TGF-β1 induced apoptosis. However, the observation that the CHX treatment resulted in lower level of the apoptosis, than seen after Dex treatment, implies that p53 may also support an apoptotic pathway independent of Bax. This notion is consistent with the observation that Actinomycin-D treatment induced p53 expression without any increase in Bax expression and resulted in extensive apoptosis (data not shown). We therefore analyzed the possible involvement of AP-1 activity in the apoptosis of the RLE cell in light of reports showing that AP-1 DNA binding factor was induced by TGF-β1 (15) and also reduced by Dex (16,17). It was revealed that TGF-β1 treatment increased AP-1 activity in contrast to the suppression of AP-1 activity seen after administration of Dex and CHX, suggesting AP-1 as a possible transducer of TGF-β1 initiated death-signaling (Fig. 3A). In an attempt to define the function of AP-1 after TGF-β1 administration, we characterized the components of AP-1 complexes. We also showed that treatment with TGF-β1 or TGF-α resulted in induction of the same AP-1 components, jun-D and fra-2 (Fig. 3B). Although these results did not suggest that the transcriptional control of Bax was induced by the newly upregulated AP-1 components, we could not exclude the possibility that jun-D and fra-2 components might be required for apoptosis, due to growth-suppressive function of jun-D

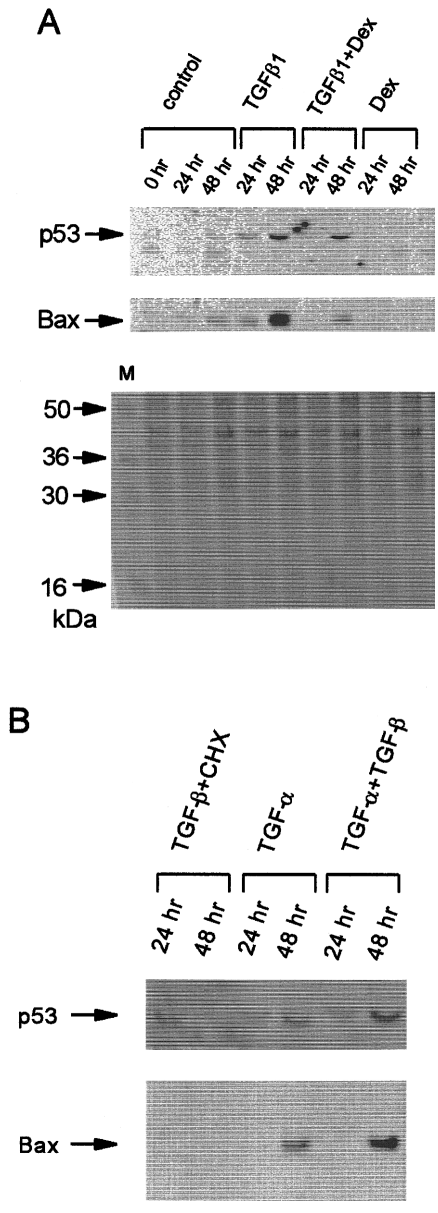

FIG. 2. Modulation of p53 and Bax expression by TGF-β1. Panel **A:** Comparison of TGF-β1 induced p53 and Bax expression and the impact of Dex treatment; Panel **B:** Regulation of p53 and Bax expression by TGF-β1 and TGF-α alone and in combination. Also inhibition of TGF-β1 induced p53 and Bax expression by CHX is shown.

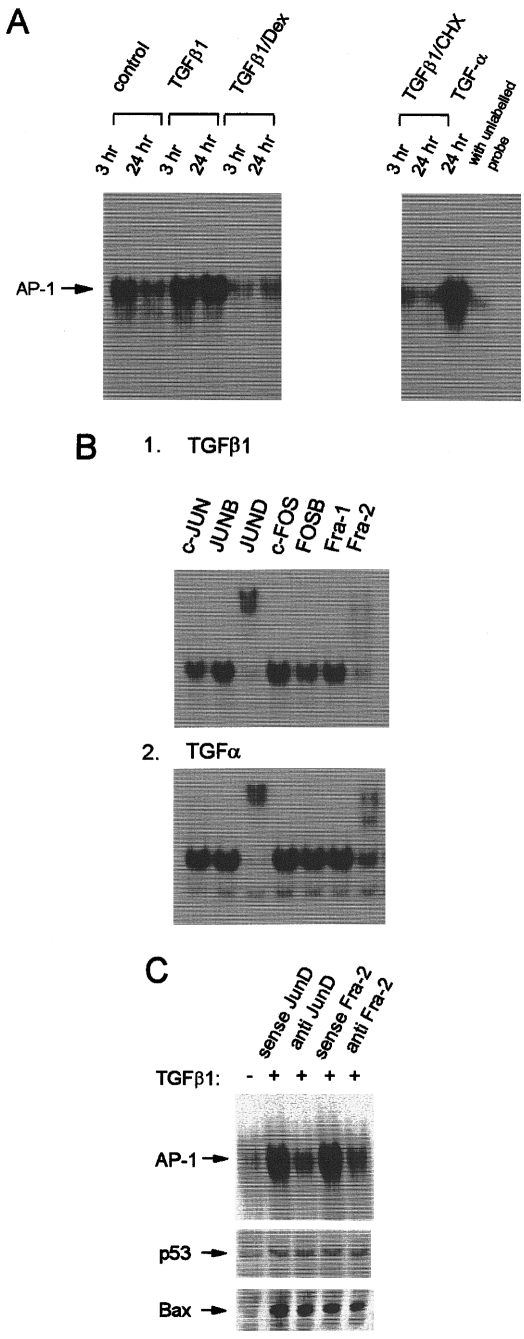

FIG. 3. Induction of AP-1 DNA binding activity in RLE cells by TGF-β1. Panel **A:** Treatment with TGF-β1 induced AP-1 activity in RLE cells. The AP-1 activity was effectively inhibited by either Dex or CHX. The gel shift assay was performed with 5μg of nuclear protein. Panel **B:** Components of the AP-1 complex induced by TGF-β1 [1] and TGF-α [2] were identified by gel supershift assay. The shifted bands for junD and fra-2 are observed. Panel **C:** Treatment of RLE cells with antisense oligonucleotides for junD and fra-2 (80 μg/ml) inhibited TGF-β1 dependent induction of AP-1 activity without affecting the induction of p53 and Bax.

(18) and the positive effect of jun-D and fra-2 on differentiation (19). We therefore designed experiments aimed at directly suppressing the jun-D and fra-2 components by using antisense oligonucleotides against these components. The result showed that the direct inhibition of AP-1 activity during TGF-β1 exposure did neither alter the rate of apoptosis nor the amounts of Bax expression (Fig. 3C). The same result was observed after TGF-α treatment (data not shown). We conclude that the induced AP-1 activity may not significantly impact the TGF-β1 induced apoptotic cascade in RLE cells.

TGF-β1 reduces NF-κB activity. It has been proposed that Dex treatment promotes TNF-α-driven apoptosis by interfering with the binding capacity of NF-κB to promoters of potential death-antagonists (20). Since this mechanism could not explain our data showing that Dex prevented the TGF-β1 induced apoptosis, we wanted to examine if the level of NF-κB activity was in some way related to the TGF-β1 induced apoptosis in the RLE cells. Basal expression level of NF-κB was very low in RLE cells, as compared with that seen in C4T cells, a transformed variant derived from RLE cells that is resistant to TGF-β1 induced apoptosis (data not shown). Furthermore, NF-κB activity in RLE cells was down-regulated after TGF-β1 treatment in presence of FBS. These data suggested the possibility that the lowered level of NF-κB activity might contribute to the apoptosis in the RLE cell. However, this possibility appears unlikely since NF-κB activity is increased after TGF-α treatment that induces apoptosis in the RLE cells (data not shown). These results suggested that the apoptosis antagonist(s) which are generally assumed to be induced by NF-κB may not be expressed in RLE cells or may not play a critical role in preventing apoptosis in the cell.

In conclusion, we have shown that TGF-β1 induced apoptosis in RLE cells occurs via the p53/Bax pathway. These results differ from those observed in TGF-β1 induced apoptosis in primary rat hepatocytes and rat hepatoma cell lines, suggesting that during apoptosis of these cells TGF-β1 signaling pathway may recruit different components of the apoptotic cascade.

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