ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb; 22 (2): 176 - 182
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Cycloheximide blocks TGF-β_1-induced apoptosis in murine hepatocytes[1]

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KEY WORDS transforming growth factor β; apoptosis; cycloheximide; ceramides

ABSTRACT

AIM: To study the mechanism of transforming growth factor β_1-induced apoptosis in cultured hepatocytes. **METHODS**: DNA fragmentation and fluorescent microscopy were used to characterize cell apoptosis. Crystal violet staining was used to assess cell viability. Immunoblotting was used to detect Tak1, p53, and Bax. Dual luciferase assay was used to determine TGF-β_1-induced gene expression. Thin layer chromatography was used to examine ceramide level in AML12 cells. **RESULTS**: In response to TGF-β_1 treatment, AML12 cells exhibited typical changes, which was characteristic of apoptosis, such as condensation of chromatin, disintegration of nuclei, and DNA fragmentation. TGF-β_1-induced apoptosis in AML12 cells was completely blocked in the presence of cycloheximide. The inhibitory effect of cycloheximide was accompanied with down-regulation of Tak1 expression and TGF-β_1-induced PAI-1 expression. TGF-β_1 induced p53 expression but not Bax. No increase of ceramide was obeserved in TGF-β_1-induced apoptosis. **CONCLUSION**: TGF-β_1-induced apoptosis requires TGF-β_1-induced gene expression.

INTRODUCTION

Transforming growth factor β (TGF-β) is a multifunctional cytokine of M_r 25 000, which plays important roles in the regulation of a wide variety of cellular processes including differentiation, proliferation, adhesion, apoptosis, and migration. Abnormality in TGF-β signaling has been implicated in some diseases, including cancer[1]. The post-receptor signaling pathway(s) induced by TGF-β in many biological processes has been a focus of study in recent years. TGF-β exerts its cellular effects by binding uo transmembrane receptors that possess serine/threonine kinase activity. Upon ligand binding, a heteromeric receptor complex consisting of two type II and two type I receptors is formed. Within the complex, the type I receptor (TGF-βR-I) is phosphorylated and activated by the constitutively active kinase of type II receptor (TGF-βR-II). The activated TGF-βR-I phosporylates the SSXS motif of downstream molecules Smad 2 and Smad 3 at the extreme carboxyl termini. The active Smads form heteromeric complexes with common mediator Smad 4 and then the complexes translocate to the nucleus to regulate gene transcription[1,2]. In other signaling pathways of TGF-β, Tak1, a MAPKKK, is responsible for the activation of ERK, JNK and p38. The three MAP kinase pathways are involved in the regulation of TGF-β-induced transcriptional activation by regulating the Smad-mediated pathway[3].

TGF-β stimulates proliferation of cells of mesenchymal origin. Whereas, it exerts potent antiproliferative effects on many other cell types, such as epithelial and endothelial cells and those of hematopoietic origin. In addition, TGF-β has been reported to induce apoptosis in several types of cells including hepatocytes and hepatomas. Thus, the escape of tumor cells from TGF-β induced growth arrest and/or apoptosis would result in an uncontrolled autonomous cell growth. It appears that the effect of TGF-β induced growth arrest is linked to the suppression of pRB phosphorylation, because TGF-β significantly induces the expression of some key regulators of cell cycle, such as p16ink4b, p21cip1, and p27kip1. However, the mechanism of TGF-β induced apoptosis still remains to be characterized. AML12 murine hepatocytes are very sensitive to TGF-β_1 and undergo apoptosis upon TGF-β_1 treatment. TGF-β_1 regulates cell responses

[1] Project supported by the Chinese Academy of Sciences (No KJ951-B1-608, "100-Project Program") and the National Natural Science Foundation (No 396250007 and 39870396).
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Received 2000-04-10 Accepted 2000-11-16

through modulation gene transcription. In present study, we try to determine the necessity of TGF-β_1-mediated gene expression in apoptotic process. We used a protein synthesis inhibitor, cycloheximide, to pretreat AML12 cells, and investigated the effects of cyclohexmide on TGF-β_1-induced apoptosis and gene expression.

MATERIALS AND METHODS

Cell culture AML12 murine hepatocytes (American Tissue Culture Collection, ATCC) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing insulin 5 mg/L, transferrin 5 mg/L, selenium 5 μg/L, dexamethasone 40 μg/L, and 10 % fetal bovine serum. All these culture materials were bought from Gibco BRL Corporation (USA). Cells were seeded in 60- or 100-mm plates. Cell cultures were maintained at 37 ℃ in a humidified atmosphere of 5 % CO_2.

Assessment of cell viability Cell viability was assessed by crystal violet in 96-well plate. Briefly, 10 000 cells per well were seeded and treated with or without TGF-β_1. The cell culture medium was removed and rinsed carefully with PBS twice. The attached viable cells were fixed with 4 % formalin for 30 min and stained with 0.5 % crystal violet for 30 min. The plates were extensively washed and dried. One hundred μL of 10 % acetic acid was added per well. Optical density (OD) was measured at 570 nm.

Morphological evaluation of apoptosis Morphological evaluation was performed as described[4]. Briefly, medium was gently removed after treatment to prevent detachment of cells. Cells were stained by acridine orange (2 mg/L) and ethidium bromide (2 mg/L) in PBS. Fluorescence was visualized immediately with a fluorescent microscope. The normal cells appeared uniformly green. Early apoptotic cells were stained green and contained bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells incorporated ethidium bromide and therefore stained orange with condensed and often fragmented nuclei.

DNA fragmentation of apoptotic cells DNA fragmentation of apoptotic cells was detected as described by Lindenboim *et al*[5]. DNA were extracted and analyzed by agarose gel electrophoresis. DNA ladders were stained with ethidium bromide, observed by a UV light source and photographically documented.

Transfections and reporter assays Reporter plasmid 3TP-lux contains a cDNA sequence coding for luciferase under the control of PAI-1 promoter. The plasmids 3TP-lux and pRL-SV40 were co-transfected into AML12 cells with LipofectAMINE (Gibco BRL, USA). Sixteen hours after transfection, the cells were treated with or without TGF-β_1 (10 μg/L) in culture medium with 0.25 % FCS. The cells were lysed and the luciferase activity was measured with dual luciferase assay system (Promega, USA) and normalized to pRL-SV40 luciferase activity.

Preparation of cell lysates and immunoblotting Cells were lysed in lysis buffer (Tris 10 mmol/L pH 7.4, edetic acid 1 mmol/L, egtazic acid 0.5 mmol/L, NaCl 150 mmol/L, 1 % Triton X-100, NaF 50 mmol/L, $Na_4P_2O_7 \cdot 10H_2O$ 10mmol/L, aprotinin 5 mg/L, leupeptin 5 mg/L and phenylmethylsulfonyl fluoride 1 mmol/L). Fifty microgram of proteins were electrophoresed in SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (HybondTM ECLTM). The membranes were blocked with 5 % skim milk in Tris-buffered saline (TBS) containing 0.1 % Tween-20 (TBS-T) and subsequently incubated with anti-Tak1, p53, and Bax antibodies (obtained from Santa Cruz biotechnology, Inc). After being washed with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated antibody. The immunoreactive bands were detected by enhanced chemiluminescent reagents ECL (Amersham).

Measurement of ceramide levels Cells were seeded in 60-mm dishes in cultured medium at 37 ℃ and 5 % CO_2 atmosphere for 24 h. The medium was refreshed and the serum concentration was lowered to 0.25 %. The cells were labeled with [^{3}H]serine (obtained from Amersham Pharmacia Biotech) for 16 h and then treated with TGF-β_1 (10 μg/L) at 37 ℃ for indicated time. The reactions were terminated and the total lipids in the organic phase were extracted as previously described[6]. Ceramide bands were scrapped from TLC plates (Whatman Inc) into scintillation vials, and eluted out of the gel by adding 500 μL of methanol and then mixed with 2.5 mL of Biodegradable Counting Scintillant (BCS, Amersham). The radioactivity was determined by liquid-scintillation spectrometry (Wallac 1409, Pharmacia).

Statistical analysis Results are presented as $\bar{x} \pm s$. For statistical analysis, student's t-test was used. Differences were considered significant at a level of $P < 0.05$.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2)
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RESULTS

TGF-β_1-induced apoptotic response in AML12 cells To investigate the effect of TGF-β_1 on AML12 cells, cells were treated with indicated concentrations of TGF-β_1 for various times. As shown in Fig 1, TGF-β_1 treatment caused a decrease in cell viability. The decreased viability was detected after a 6-h treatment with TGF-β_1 (1 μg/L). However, maximum inhibition of cell viability was observed after a 48-h treatment with TGF-β_1 (10 μg/L). Fig 2a showed that TGF-β_1 induced DNA fragmentation in AML12 cells. AML12 cells were grown in 100-mm plate. At 80 % confluence, cells were treated with indicated concentrations of TGF-β_1 for 24 h (left panel), or treated with 10 μg/L of TGF-β_1 for the indicated times (right panel). TGF-β_1 10 μg/L induced pronounced DNA fragmentation, which became easily detectable after 12 h treatment. Fig 2b demonstrated the TGF-β_1-induced cell apoptosis as detected by fluorescent staining assay, which showed the chromatin condensation and fragmentation of nuclei.

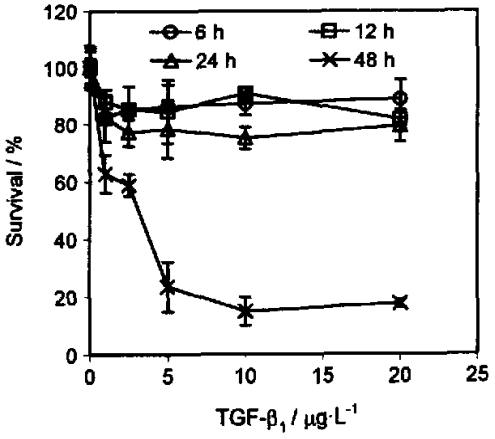

Fig 1. TGF-β_1-induced cell death in AML12 cells. Cells were treated with indicated concentrations of TGF-β_1 for the time indicated. Cell viability was determined by crystal violet assay. $n=4$. $\bar{x} \pm s$.

Cycloheximide blocked the TGF-β_1-induced apoptosis Cycloheximide, a protein synthesis inhibitor, was able to inhibit the TGF-β_1-induced apoptosis in AML12 cells. Fig 3a showed that cycloheximide inhibited TGF-β_1-induced cell death in a dose-dependent manner. In the presence of cycloheximide 0.1 mg/L, TGF-β_1-induced cell death was inhibited by 50 %. At the concentration of 1 mg/L, TGF-β_1-induced cell death was inhibited by more than 80 %. Fig 3b demonstrated the effect of cycloheximide on TGF-β_1-induced DNA fragmentation in AML12 cells. In this experiment, AML12 cells were treated with TGF-β_1 (10 μg/L) in the presence of different concentrations of cycloheximide. Cytosolic DNAs were prepared and separated by agarose gel electrophoresis. TGF-β_1-induced DNA fragmentation was completely blocked by cycloheximide. The results suggest that TGF-β_1-induced cell apoptosis requires the synthesis of new proteins.

Cycloheximide inhibited TGF-β_1-induced PAI-1 promoter activity To investigate whether the inhibition of TGF-β_1-induced apoptosis by cycloheximide resulted from the inhibition of TGF-β_1-induced gene expression, we determined the effect of cycloheximide on the activity of PAI-1 promoter. Activation of PAI-1 promoter is an event induced by TGF-β_1 signaling, which has been used as an indication for the transcriptional activity induced by TGF-β1. 3TP-lux plasmid contains a luciferase expression unit under control of PAI-1 promoter. 3TP-lux reporter gene was introduced into AML12 cells with pRL-SV40 plasmid. After 16 h of transfection, cells were treated with TGF-β_1 10 μg/L in the presence of indicated concentrations of CHX. TGF-β_1 10 μg/L induced 5.6-fold increase in luciferase activity as compared with respective nontreated controls. TGF-β_1 induced PAI-1 promoter activity was significantly inhibited by cycloheximide. In the presence of cycloheximide 1 mg/L, TGF-β_1-induced luciferase activity was reduced to 2.1-fold (Fig 4). The data indicated that TGF-β_1-induced gene expression was required for the TGF-β_1-induced cell apoptosis.

Cycloheximide reduced the level of Tak1 proteins To further investigate the involvement of possible signaling components involved in the inhibition of TGF-β_1-induced apoptosis by cycloheximide, we first detected the effects of cycloheximide on protein levels of Bax and p53, which are proapoptotic molecules and have been implicated in some apoptotic events. Cells were treated with TGF-β_1 10 μg/L and/or CHX 1 mg/L for 24 h. The expression of Bax or p53 was determined by immunoblotting. As shown in Fig 5a, both TGF-β_1 and cycloheximide increased the expression of p53, but not Bax. Cycloheximide had neither additive nor obvious inhibitory effect on TGF-β_1-induced p53 expression, suggesting that Bax and p53 are not involved in the cycloheximide-mediated inhibition of apoptosis induced by TGF-β_1. We next examined the effect of cycloheximide on the expression of Tak1. Tak1 is a MAPKKK, which

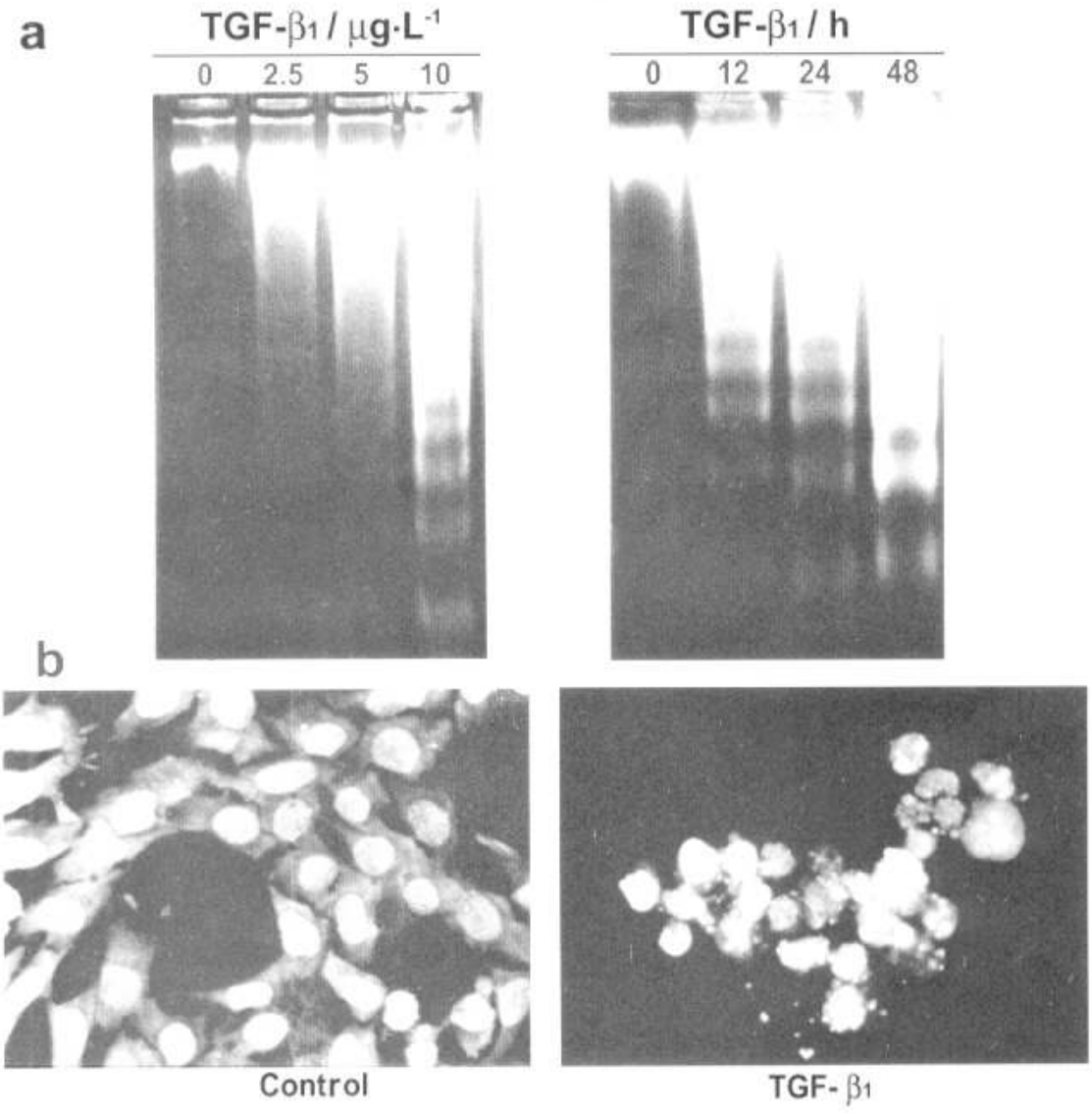

Fig 2. Characterization of TGF-β_1-induced apoptosis in AML12 cells. a) TGF-β_1-induced DNA fragmentation in AML12 cells. b) Typical apoptotic morphology was observed in cells treated with TGF-β_1 (10 μg/L) for 48 h (× 400).

can be activated by TGF-β_1. Tak1 mediates TGF-β_1 action through activation of p38 and JNK MAPK signaling pathways[3]. As shown in Fig 5b, treatment of cells with cycloheximide decreased the cellular level of Tak1 protein. The data suggest that Tak1 are required in TGF-β_1-induced cell apoptosis and therefore inhibition of Tak1 expression by cycloheximide results in an inhibition of apoptotic response to TGF-β_1 treatment.

Ceramide level in AML12 Ceramide is an early signal that is involved in apoptosis induced by some extracellular agonists, such as TNF-α and Fas. To investigate whether ceramide is involved in TGF-β_1-induced apoptosis, we determined the ceramide level in AML12 cells treated with and without TGF-β_1. As shown in Fig 6, no increase in ceramide level was observed in cells treated with TGF-β_1, suggesting that ceramide was not involved in TGF-β_1-induced cell apoptosis.

DISCUSSION

TGF-β acts as a negative regulator of cell proliferation by arresting cells in G1 phase. TGF-β also promotes cell apoptosis. The mechanism of apoptotic effect of TGF-β seems different from its anti-proliferative effect.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2)
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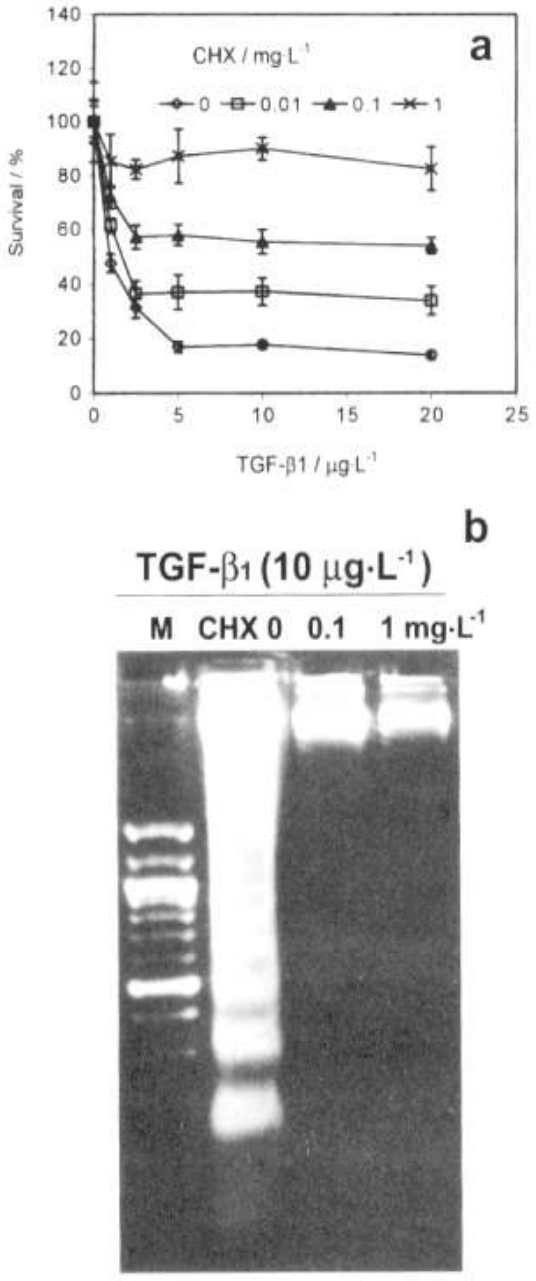

Fig 3. Cycloheximide (CHX) inhibits TGF-β_1-induced apoptosis in AML12 cells. a) Cells were treated with indicated concentrations of TGF-β_1 for 48 h in the presence of indicated concentrations of CHX. Cell viability was determined by crystal violet assay. $n=4$. $x \pm s$. b) CHX inhibited the TGF-β_1-induced DNA fragmentation in AML12 cells. M: 100 bp DNA molecular weight marker.

It has been reported that reactive oxygen species (ROS) production and caspases play a role in TGF-β-induced apoptosis. Contradictory evidence regarding the involvement of expression of apoptosis-related proteins has been

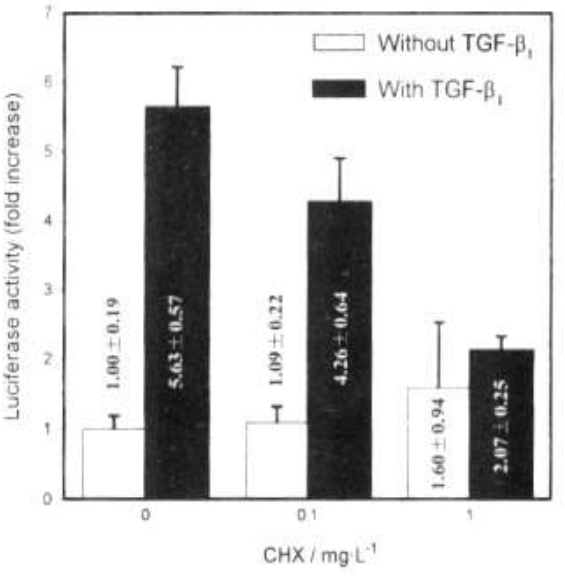

Fig 4. Cycloheximide (CHX) inhibits TGF-β1-induced activation of PAI-1 promoter in AML12 cells. The activity of luciferase was detected with dual luciferase assay system. $n=3$. $x \pm s$.

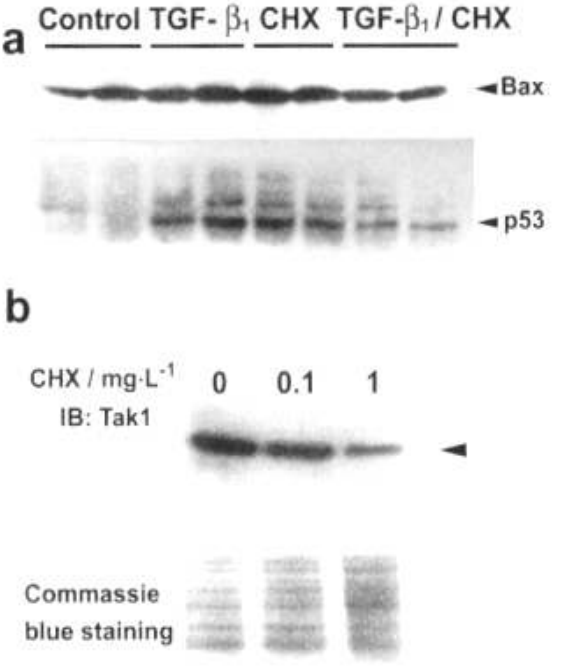

Fig 5. The effect of cycloheximide (CHX) on the expression of Bax, p53, and Tak1 in AML12 cells. a) The effect of CHX and TGF-β_1 on the expression of Bax and p53. b) CHX reduced the expression of Tak1. Cells were treated with CHX 0.1 and 1 mg/L for 24 h. Top panel shows the expression of Tak1 detected by immunoblotting and lower panel represents the total protein levels visualized by Commassie blue staining.

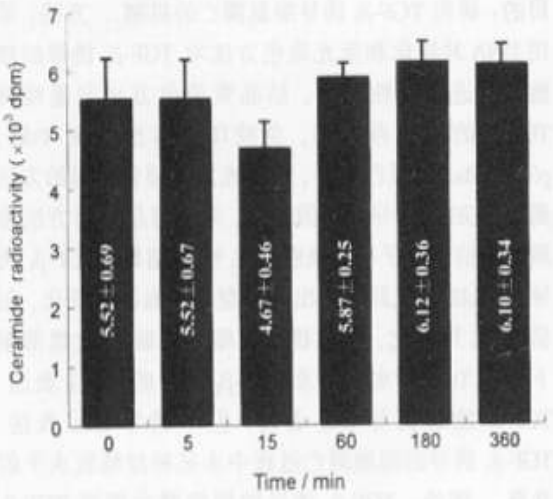

Fig 6. Effect of TGF-β_1 on ceramide formation in AML12 cells. Cells were seeded in 35-mm plates. At 80 % confluence, cells were labeled with [^{3}H]serine for 16 h, then treated with TGF-β_1 (10 μg/L) for indicated time at 37 ℃. The radioactivity of [^{3}H]serine-labeled ceramide was determined by liquid-scintillation spectrometry. $n = 3$. $\bar{x} \pm s$.

reported by several groups. Teramoto reported that TGF-β induced the expression of proapoptotic molecules p53 and Bax[7]. However, it was also reported that TGF-β did not induce any increase in the expression of Bax and Bad, but decreased the expression of Bcl-XL[8].

Our study demonstrated that TGF-β_1 induced a strong apoptosis in AML12 cells. TGF-β_1-induced apoptosis in AML12 cells was associated with characteristic condensation of chromatin, internucleosomal DNA cleavage, and could be visualized as a DNA ladder consisting of fragments that are multiples of 180 – 200 bp. Cycloheximide, an inhibitor of protein synthesis, has been shown to prevent cell from apoptotic response induced by some stimuli. In this study, we investigated the role of gene expression and protein synthesis in TGF-β_1-induced apoptosis. We found that cycloheximide strongly inhibited TGF-β_1-induced apoptosis in AML12 cells, suggesting that TGF-β_1-induced gene expression or protein synthesis is required in its apoptotic effect. The conclusion was supported by the observation that the induction of PAI-1 promoter activity by TGF-β_1 was also abolished by cycloheximide. The evidence that cycloheximide down-regulated Tak1 indicates that Tak1 plays a role in the apoptotic effect of TGF-β_1. TGF-β_1-induced expression of p53 is probably a required but not sufficient factor involved in TGF-β_1-induced apoptosis in AML12 cells because cycloheximide itself can increase p53 expression.

Tan *et al*[9] showed that Hematopoietic progenitor kinase 1 mediates the TGF-β-induced activation of Tak1 and JNK. However, the activation of Tak1 by TGF-β remains to be further identified. It has been reported recently that Tak1 mediates ceramide-induced activation of JNK[10]. We therefore determined whether ceramide was involved in TGF-β_1-induced apoptosis. Our results showed that no increase in ceramide level was induced in response to TGF-β_1 treatment, suggesting that ceramide is unlikely to be involved in the TGF-β_1-induced apoptosis in AML12 cells.

In summary, the data presented in this report indicate that TGF-β_1-induced apoptosis in AML12 cells requires protein synthesis and TGF-β_1-induced gene expression. The results also imply that Tak1 is likely a signaling molecule involved in the TGF-β_1-induced apoptosis.

ACKNOWLEDGEMENT We are grateful to Dr Rik Derynck for providing TGF-β_1 and Dr Joan Massagué for providing the PAI reporter plasmid 3TP-lux.

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放线菌酮抑制转化生长因子-β_1 诱导的小鼠肝细胞的凋亡[1]

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关键词 转化生长因子 β；细胞凋亡；放线菌酮；神经酰胺类

目的：研究 TGF-β_1 诱导细胞凋亡的机制. **方法**：采用 DNA 片段化和荧光染色方法对 TGF-β_1 诱导的细胞凋亡进行定性观察，结晶紫染色方法定量检测 TGF-β_1 的细胞存活率，免疫印迹方法检测 Tak1、p53 和 Bax 的蛋白水平，用荧光素酶报告基因的方法测定 TGF-β_1 诱导的基因表达，采用薄层层析方法检测脂类信号分子神经酰胺的水平. **结果**：TGF-β_1 诱导 AML12 小鼠肝细胞出现典型的细胞凋亡变化，包括 DNA 片段化，细胞核的固缩、碎裂. 放线菌酮下调了 Tak1 的水平以及 TGF-β_1 诱导的 PAI-1 表达. TGF-β_1 能够诱导 p53 表达，但不诱导 Bax 表达. TGF-β_1 诱导的细胞凋亡过程中未见神经酰胺水平的升高. **结论**：TGF-β_1 诱导的细胞凋亡需要 TGF-β_1 诱导的基因表达.

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