TGF- β 1 suppresses apoptosis via differential regulation of MAP kinases and ceramide production

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Abstract

Serum deprivation induces apoptosis in NIH3T3 cells, which is associated with increased intracellular ceramide generation and with the activation of p38 mitogen-activated protein (MAP) kinase. Treatment of cells with transforming growth factor- β 1 (TGF- β 1) activated the extracellular signal regulated kinases 1 and 2 (ERK1/ERK2), inhibited the serum deprivation-induced p38 activation and the increase in intracellular ceramide formation, leading to the stimulation of cell proliferation and the suppression of apoptosis. Inhibition of p38 MAP kinase by SB203580 significantly reduced the serum-deprivation-induced apoptosis. Overexpression of p38 increased the cell apoptosis and reduced the antiapoptotic effect of TGF- β 1. Inhibition of ERK1/ERK2 by PD98059 completely inhibited the TGF- β 1-stimulated proliferation and partially inhibited the antiapoptotic effects of TGF- β 1. Neither SB203580 nor PD98059 has obvious effect on TGF- β 1-mediated inhibition of the increased ceramide generation. Serum-deprivation-induced apoptosis in NIH3T3 cells can also be blocked by broad-spectrum caspase inhibitor. TGF- β 1 treatment has an inhibitory effect on caspase activities. Our results indicate that ceramide, p38, and ERK1/ERK2 play critical but differential roles in cell proliferation and stress-induced apoptosis. TGF- β 1 suppresses the serum-deprivation-induced apoptosis via its distinct effects on complex signaling events involving the activation of ERK1/ERK2 and the inhibition of p38 activation and increased ceramide generation.

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Abbreviations: TGF- β 1, transforming growth factor- β 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SMase, sphingomyelinase

Introduction

The balance between cell proliferation, differentiation, and apoptosis is controlled by various internal and external stimuli, which play crucial roles in normal development and homeostasis of living cells and organisms. Disruption of this balance by different factors may lead to abnormal cell death, growth, and many pathological events, including cancer, immune and developmental diseases. Studies on the underlying mechanisms through which the balance between cell proliferation, differentiation, and apoptosis are maintained or disrupted are important in understanding various physiological and pathological processes. It is now known that complex signaling network, which is formed by the interaction or 'crosstalk' among various signaling molecules and pathways, is implicated in the subtle and general control of cell proliferation, differentiation, and apoptosis.

Intracellular second messengers are generated in the course of signal transduction or various cellular processes. They function as mediators in controlling the survival, proliferation, and apoptotic events via different or similar signaling mechanism. The production of ceramide, a potent bioactive sphingolipid derivative, can be stimulated by stress, including tumor necrosis factor- α (TNF- α), chemotherapeutic reagents, and the deprivation of survival signals.^{1–4} Increased intracellular ceramide formation in response to the above stimuli is followed by apoptotic and nonapoptotic cell death. Exogenous cell permeable ceramide analogs, C₂- and C₈-ceramide, are also able to induce apoptosis in a variety of cells.

Mitogen-activated protein (MAP) kinase, a large family of protein kinases, plays pivotal roles in controlling the cellular behavior or functions. MAP kinase can be regulated by many upstream signaling events, and therefore may serve as an integrator for diverse signals or signaling pathways. The MAP kinase activity has been shown coupled with numerous biological events induced by various stimuli.^{5–7} Different biological effects may be produced by activation or inhibition of different MAP kinases; likewise, down- or upregulation of the same MAP kinase activity may induce opposite biological effects, which suggests that the specific effects of MAP kinase may be induced in accordance with the cellular context and their environment.

TGF- β is a pleiotropic polypeptide that involves in both the negative and positive regulations of cell proliferation, differentiation, immune responses, and apoptotic processes.^{8,9} TGF- β and its mediated signaling pathways, which play critical roles in the formation of complex signaling network, have been a major focus of extensive studies.¹⁰ The biological effect of TGF- β depends on the cell types and the environmental and physiological conditions of cells. It has been reported that abrogation of TGF- β signaling in T cells leads to spontaneous T-cell differentiation and autoimmune disease,^{11,12} indicating that TGF- β signaling is required for the

maintenance of the T-cell homeostasis. Disruption of TGF- β signaling has been detected in some tumors or cancer cells. TGF- β has a strong inhibitory effect on the proliferation of epithelial cells.¹³ A growing number of evidence has shown that TGF- β is implicated in various diseases. The escape of cells from TGF- β -induced growth arrest and/or apoptosis would result in an uncontrolled, autonomous cell growth.^{14,15} On the other hand, TGF- β has an antiapoptotic function, which increases the survival rate of cells.^{16,17} TGF- β suppresses the differentiation of fibroblast cells into fat cells.^{18–21} The involvement of Smad protein in TGF- β -mediated suppression of adipogenesis has been reported.²² TGF- β has also been shown to be a stimulator for cell proliferation and differentiation.^{23,24} In addition, TGF- β has been reported to induce apoptosis in several types of cells including hepatocytes and hepatomas.^{25–28} Studying the signaling mechanism through which the diverse or antagonistic effects of TGF- β were mediated is therefore very important for a better understanding of some life phenomena and for providing necessary theoretic basis in relevant potential medical practices. The mechanism of TGF- β -mediated signaling on the receptor level has been well illustrated. Receptor-downstream signaling events transmitted by Smads and their interaction has been intensively studied and characterized in the past several years.^{10,29} The ERK, JNK, and p38 MAP kinases are found in recent years to be involved in the regulation of TGF- β -mediated signaling events.^{7,14} Crosstalk between different signaling components of TGF- β signaling pathway may play a key role in the specificity of TGF- β -mediated diverse effects.

Although a rapid progress is being made on the diverse effects and the underlying mechanisms mediated by TGF- β , relatively very little is known about its signaling mechanism of the apoptotic, antiapoptotic, and the growth stimulation functions. In this report, we studied the effect of TGF- β 1 on serum-deprivation-induced apoptosis and on the proliferation in NIH3T3 cells. Inhibition of the proliferation of NIH3T3 cells by deprivation of serum is correlated with an apoptotic response that became apparent 15h after the serum withdrawal. Serum-deprivation-induced apoptosis in NIH3T3 cells is associated with caspase activity and persistent activation of p38 MAP kinase. Treatment of cells with TGF- β 1, caspase inhibitor, or inhibition of p38 MAP kinase by SB203580, suppressed the serum-deprivation-induced apoptosis. Overexpression of wild-type p38 MAP kinase increased the cell apoptosis and reduced the antiapoptotic effect of TGF- β 1, whereas the overexpression of mutant p38 MAP kinase reduced the cell apoptosis. TGF- β 1-mediated suppression of serum-deprivation-induced apoptosis is correlated with the activation of ERK1/ERK2 and the inhibition of p38 MAP kinase and caspase activity. This antiapoptotic effect of TGF- β 1 can be blocked or enhanced by inhibiting ERK1/ERK2 and p38 MAP kinase, respectively. We also found that serum-deprivation-induced apoptosis of NIH3T3 cells is associated with an increase in intracellular ceramide level, which can also be suppressed by TGF- β 1. Our results provided new evidence that may be interesting and insightful in the understanding of the mechanism of the roles of TGF- β 1 in cellular survival and apoptosis.

Results

TGF- β 1 induces proliferation and prevents serum-deprivation-induced apoptosis in NIH3T3 cells

We observed that deprivation of serum in culture medium induces apoptosis of NIH3T3 cells. Before determining the effect of TGF- β 1 on serum-deprivation-induced apoptosis, we firstly examined its effect on the growth of NIH3T3 cells. Incubate cells with TGF- β 1 for different times induces the DNA synthesis as measured by [³H]-thymidine incorporation into NIH3T3 cells, which is pronounced after treatment for 16 h (Figure 1a) and in a concentration-dependent manner (Figure 1b). At 5 ng/ml of TGF- β 1, DNA synthesis of cells incubated in the absence of serum increased up to 10-fold after 24 h. Serum deprivation in culture medium induces morphological alterations characteristic of apoptotic cellular response, such as cell shrinkage, chromatin condensation,

Figure 1 TGF- β 1 stimulates the proliferation of NIH3T3 cells. Cells in 24-well plates were grown to 80% confluence and then incubated in a medium containing 5 or 0.5% NCS and were treated with TGF- β 1. (a) Time course of TGF- β 1-stimulated [³H]-thymidine incorporation into NIH3T3 cells. Cells were treated with TGF- β 1 (5 ng/ml) for different times. DNA synthesis was determined as described in the Materials and Methods section. The average cpm for controls was 137 (0.5% NCS) and 464 (5% NCS), respectively. (b) Dose-dependent effect of TGF- β 1 on cell proliferation. DNA synthesis was determined after cells were treated with indicated concentrations of TGF- β 1 for 24 h. The average cpm of controls, 151 (0.5% NCS) and 457 (5% NCS), respectively, was set as 100%. Data are the means $\pm$ S.D. of three experiments performed in triplicates

nuclear and DNA fragmentation, which were detected by fluorescent staining, DNA fragmentation, and FACS assay (Figure 2). Apoptosis detected by DNA fragmentation assay became highly prominent 15 h after serum deprivation (Figure 2a). The presence of TGF- β 1 in the serum-deprived medium protected cells from apoptosis as observed by either

fluorescent staining (Figure 2b) or DNA fragmentation assay (Figure 2c), indicating that TGF- β 1 is an important surviving and antiapoptotic factor for NIH3T3 cells. TGF- β 1-mediated inhibition of apoptosis is dose dependent, which became evident at a concentration of 5 ng/ml (Figure 2c, left panel). Addition of TGF- β 1 8 h after withdrawal of serum still inhibits,

but in an obviously much reduced extent the cell apoptosis (Figure 2c, right panel), which is in correlation with the kinetics of apoptotic events undergoing in NIH3T3 cells in response to serum deprivation. We also determined by FACS assay the TGF- β 1-mediated suppression of serum-deprivation-induced apoptosis at different time points. As shown in Figure 2d, e, TGF- β 1 remains highly effective in the suppression of serum-deprivation-induced apoptosis that lasts for as long as 48 h. A markedly decrease in this antiapoptotic function of TGF- β 1 occurred 3 days (72 h) after serum withdrawal even if the TGF- β 1 was supplemented once more at the time point of 48 h. At the same time point, cell apoptosis in serum-starved culture (without TGF- β 1) increased to approximately 52%. Apoptosis of cells incubated in the presence of serum also showed an obvious increase (up to approximately 11%), which may result from a gradual exhaustion of multiple surviving or growth-stimulating factors existed in the serum.

To determine the involvement of caspases in serum-deprivation-induced apoptosis, we examined the effect of caspase inhibitor on serum-deprivation-induced apoptosis, we also examined the effect of TGF- β 1 on the caspase activity in NIH3T3 cells. As it was shown in Figure 2f (left panel), the broad-spectrum caspase inhibitor strongly suppressed the serum-deprivation-induced apoptosis as determined by DNA fragmentation assay, indicating that caspases activation is implicated in the serum-deprivation-induced apoptosis. Treatment of cells with TGF- β 1 resulted in the suppression of serum-deprivation-induced activities of caspases in NIH3T3 cells as determined by an *in vitro* assay (Figure 2f, right panel).

TGF- β 1 inhibits serum-deprivation-induced ceramide generation

Since ceramide has been implicated in cell apoptosis induced by various factors, we examined the effect of serum deprivation on the intracellular ceramide levels. Withdrawal of serum in cell culture causes an increase in intracellular ceramide level in NIH3T3 cells (Figure 3a), which became pronounced after 12 h and showed no decline at the time point of 24 h. No ceramide increase occurred in cells cultured in a medium containing 5% new born calf serum (NCS).

Treatment of cells with TGF- β 1 blocked serum-deprivation-induced ceramide generation. This suppressive effect of TGF- β 1 on ceramide formation correlates with its antiapoptotic function on NIH3T3 cells cultured under the same conditions. Fumonisin B1 (FB1), an inhibitor for ceramide synthase in ceramide *de novo* synthesis, showed no effect on the serum-deprivation-induced ceramide generation (Figure 3b). Treatment of cells with FB1, significantly reduced the background level of intracellular ceramide, which causes a proportional reduction of ceramide level in cells deprived of serum both in the presence or absence of TGF- β 1. *N*-oleoyl-ethanolamine (NOE), a ceramidase inhibitor, that can increase basal level of intracellular ceramide in A-431 cells (Figure 3b, right panel), also has no effect on the basal level of ceramide and the ceramide production mediated by serum deprivation or TGF- β 1 in NIH3T3 cells. The data indicate that serum-deprivation-induced increase in ceramide formation is not via the *de novo* synthesis pathway or via decreased degradation of intracellular ceramide. TGF- β 1 is able to suppress the serum-deprivation-induced ceramide generation and the corresponding apoptotic response. However, TGF- β 1 has no obvious effect on exogenous cell permeable ceramide analog C₈-ceramide-induced cell apoptosis (Figure 3c).

In line with increased ceramide formation in response to serum deprivation, there is a decrease in the cellular level of sphingomyelin (SM) (Figure 4a), suggesting that increased ceramide may be generated via SMase-catalyzed hydrolysis of SM. In addition, increases in acidic and neutral (Mg²⁺-dependent and -independent) SMase activities in response to serum deprivation were observed by an *in vitro* assay (Figure 4b–d). Treatment of cells with TGF- β 1 completely inhibited the serum-deprivation-induced acidic and neutral (Mg²⁺-dependent) SMase activities as well as the background levels of SMase activities. The inhibitory effect of TGF- β 1 on neutral Mg²⁺-independent SMase activity appears to be weaker than its effects on the other two SMase activities.

TGF- β 1 activates ERK1/ERK2 and inhibits serum-deprivation-induced activation of p38 MAP kinases

MAP kinase signaling pathway has been implicated in many physiological and pathological processes. To further investigate

Figure 2 TGF- β 1 suppresses the serum-deprivation-induced apoptosis of NIH3T3 cells. Cells cultured in 60-mm plates were grown to 80% confluence, then deprived from serum and incubated in a medium containing 0.5% BSA for the times indicated in the presence or absence of TGF- β 1. Apoptosis was examined by DNA fragmentation, fluorescence staining, and FACS assay as described. (a) Cells were deprived from serum for indicated times, the apoptotic response of cells was then determined by examining the DNA fragmentation. (b) Cells cultured in 35-mm plates were incubated in a medium with or without 5% NCS in the presence or absence of TGF- β 1 (5 ng/ml) for 24 h. Apoptosis of cells was determined by AO/EB staining that showed the cell nuclear condensation and fragmentation. (c) Cells were incubated in serum-deprived medium for 24 h in the presence or absence of TGF- β 1. Cell apoptosis was determined by DNA fragmentation assay. Left panel: dose effect of TGF- β 1 on serum-deprivation-induced apoptosis; right panel: TGF- β 1 (5 ng/ml) was added at different times before the termination of incubation. (d) Cells cultured in 35-mm plates were deprived or not deprived of serum in the presence or absence of TGF- β 1 (5 ng/ml) and further incubated for the indicated times. TGF- β 1 (5 ng/ml) was added afresh 48 h after incubation. Apoptosis of cells was determined by FACS assay. Data represent one of at least four experiments with the same results. (e) Statistic presentation of (d) which are the means $\pm$ S.D. of four experiments. (f) Left panel: effect of caspase inhibitor on serum-deprivation-induced apoptosis. Cells (80% confluence) cultured in 60-mm plates were incubated in the serum-free medium containing 0.5% BSA for additional 15 h in the presence or absence of broad-spectrum caspase inhibitor III of indicated concentrations. Cell apoptosis was examined by DNA fragmentation assay. Cas III, Caspase inhibitor III. Data represent one of the three experiments with same results. Right panel: TGF- β 1 inhibits serum-deprivation-induced caspase activity. Confluent cells (80%) were incubated in serum-free or serum-containing medium in the presence or absence of TGF- β 1 (5 ng/ml) or caspase inhibitor III (10 μ M, Cas III) for 15 h. Caspase activity was examined as described in the Materials and Methods section. Casp-1 IV, Caspase-1 substrate IV; Casp-1 VII, Caspase-1 substrate VII; Casp-3 I, Caspase-3 substrate I; Casp-6 II, Caspase-6 substrate II; Casp-8 I, Caspase-8 substrate I. Data are means $\pm$ S.D. of five experiments

Figure 3 Intracellular ceramide formation. (a) TGF- β 1 inhibits serum-deprivation-induced ceramide formation. Cells (70% confluence) were labeled with [3 H]-serine (4 μ Ci/ml) overnight in normal growth medium and further incubated in serum-deprived medium or in the medium containing 5% NCS (control) for indicated times in the presence or absence of TGF- β 1 (5 ng/ml). Serum-deprivation-induced ceramide formation (—●—) and TGF- β 1-mediated suppression of serum-deprivation-induced ceramide formation (—○—) were determined. Ceramide was extracted and measured as described in the Materials and Methods section. (b) Effect of ceramide synthase inhibitor FB1 and ceramidase inhibitor NOE on the ceramide formation in NIH3T3 cells. [3 H]-serine-labeled cells were incubated in serum-deprived medium and treated with FB1 (10 μ M) or NOE (50 μ M) in the presence or absence of TGF- β 1 (5 ng/ml). The production of intracellular ceramide was determined after 24 h. (c) The effect of TGF- β 1 on ceramide-induced apoptosis. Confluent cells (50%) in 35-mm plates were incubated in 1% NCS and treated with exogenous cell permeable C $_8$ -ceramide (25 μ M) for 24 h in the presence or absence of TGF- β 1 (5 ng/ml). Apoptosis was determined by AO/EB staining. Data (a and b) are means $\pm$ S.D. of three experiments performed in duplicates; data in (c) represent one of the three experiments with same results

the mechanism of antiapoptotic function of TGF- β 1, we studied the involvement of MAP kinase in the suppression of apoptotic response to serum deprivation in NIH3T3 cells by TGF- β 1. Cells were grown to confluence and then further incubated in serum-deprived medium for indicated times in the absence or presence of TGF- β 1 added at the different time points during the incubation. As shown in Figure 5a, in response to TGF- β 1 treatment, there is a gradual but sustained increase in phosphorylated forms of ERK1/ERK2 that does not decline at the time point of 16 h during the incubation, indicating that TGF- β 1 induces a rapid and continued activation of ERK1/ERK2. Serum deprivation

reduced the levels of phosphorylated forms of ERK1/ERK2 at an early stage, but showed no obvious effect after 2 h (Figure 5b). In contrast, a gradual but sustained activation of p38 MAP kinase was induced in response to serum deprivation (Figure 5c). The increase in phosphorylated p38 is an event that can be detected as early as 20 min after serum withdrawing and showed no tendency of decline after 24 h. Addition of TGF- β 1 in the serum-deprived medium inhibited the activation of p38 as shown by the decrease in the phosphorylated form of p38 MAP kinase (Figure 5d). No obvious inhibition of p38 activation was exhibited when TGF- β 1 was added 16 h after serum withdrawal, which further suggests that p38 activation is an early signaling event generated in the serum-deprivation-induced apoptosis. Cell apoptosis was further determined after cells were transfected with wild-type or mutant p38 MAP kinase, in order to confirm the effect of p38 in serum-deprivation-induced apoptosis. Transfection of wild-type p38 not only leads to an increase in p38 level, but also causes an increase in the background level of phosphorylated p38 (Figure 6a), which resulted in a significant increase in the levels of basal and serum-deprivation-induced apoptosis and a corresponding decrease in the antiapoptotic effect of TGF- β 1 (Figure 6b). In contrast, transfection of cells with mutant p38 MAP kinase reduced the serum-deprivation-induced apoptosis. The evidence further supports the role of p38 in apoptotic and antiapoptotic processes mediated by serum deprivation and TGF- β 1.

Inhibition of ERK1/ERK2 and p38 MAP kinases causes opposite effects on apoptosis in NIH3T3 cells

To further confirm the roles of MAP kinase in cell apoptosis, we use SB203580, a specific inhibitors of p38, and PD98059 that specifically inhibits MEK (MAPK/ERK kinase) and consequently results in the inhibition of ERK1/ERK2, to investigate the effects of ERK1/ERK2 and p38 MAP kinase on serum-deprivation-induced apoptosis. Treatment of cells with either TGF- β 1 or SB203580 strongly inhibited serum-deprivation-induced apoptosis as determined by DNA fragmentation (Figure 7a). The combinatory use of TGF- β 1 and SB203580 leads to a more complete suppression of serum-deprivation-induced apoptosis, which can be reduced to below the basal level. The results provided additional support on the role of p38 MAP kinase in serum-deprivation-induced apoptosis, and suggested that p38 activation induced by serum deprivation is sufficient to provoke apoptotic response of cells. The data also indicate that the antiapoptotic effect of TGF- β 1 in NIH3T3 cells is through a mechanism involving the inhibition of p38 MAP kinase. In contrast, treatment of cells with PD98059 moderately suppressed the TGF- β 1-mediated inhibition of DNA fragmentation (Figure 7b). By fluorescent staining assay, we further confirmed the effects of p38 and ERK1/ERK2 MAP kinases in serum-deprivation-induced apoptosis (Figure 7c). Serum deprivation causes morphological alterations such as nuclear condensation and chromatin fragmentation, which is characteristic of apoptosis, was completely inhibited by treatment of cells with selective p38 MAP kinase inhibitor SB203580, whereas TGF- β 1-mediated

Figure 4 TGF- β 1 inhibits the SMase activities and the decrease in SM level induced by serum deprivation. (a) TGF- β 1 inhibits the serum-deprivation-induced decrease in SM level. [3 H]-serine-labeled NIH3T3 cells were incubated in serum-deprived medium for 12 or 24 h in the presence and absence of TGF- β 1 and the SM was measured. The cpm of SM from nontreated cells is 1034.7. *, **, and *** are significance of differences between the indicated bar charts at levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. (b–d) TGF- β 1 inhibits the SMase activities. Confluent cells (80%) were cultured in the medium containing 0.5% BSA in the presence or absence of TGF- β 1 (5 ng/ml) for the different times, then further treated for assaying SMase activities as described in the Materials and Methods section. (b) The acidic SMase activity. (c) Mg^{2+} -dependent neutral SMase activity. (d) Mg^{2+} -independent neutral SMase activity. The average cpm of controls for (b), (c), and (d) was 488, 648, and 292, respectively. Data are the means $\pm$ S.D. of three experiments performed in duplicates

suppression of apoptosis was less effectively blocked by MEK inhibitor PD98059. The effects of p38 and ERK1/ERK2 MAP kinases were also investigated by FACS assay and similar results were obtained. As shown in Figure 7d, PD98059 partly inhibited the TGF- β 1's antiapoptotic effect. Conversely, SB203580 inhibited serum-deprivation-induced apoptosis and exhibited an additive effect on the antiapoptotic effect of TGF- β 1. SB203580 can significantly influence the serum-deprivation-induced cell apoptosis; however, it has no effect, as shown in Figure 7e, on the serum deprivation-induced ceramide generation, or does it affect the TGF- β 1-mediated inhibition of ceramide formation.

Since TGF- β 1 is able to stimulate the proliferation of NIH3T3 cells, we investigated by the possible involvement of p38 and ERK1/ERK2 MAP kinases in TGF- β 1-mediated proliferation of NIH3T3 cells. As shown in Figure 8a, TGF- β 1-stimulated proliferation of NIH3T3 cells can be completely blocked by PD98059. In contrast, SB203580 itself has no effect on the proliferation of NIH3T3 cells; it significantly enhanced the TGF- β 1-stimulated proliferation of NIH3T3 cells (Figure 8b).

Discussion

The pivotal role of TGF- β signaling in the control of cell growth, differentiation, and death has been demonstrated by mounting evidence. Nevertheless, the signaling mechanism downstream the receptor level underlying its various effects remains largely unidentified, especially the mechanism of pro- and antiapoptotic functions of TGF- β is poorly understood. In this report, we investigated primarily the antiapoptotic effect and potential mechanism of TGF- β 1 on the serum-deprivation-induced cell apoptosis. We also examined the regulatory effect of TGF- β 1 on the proliferation of NIH3T3 cells. TGF- β 1 treatment increases the proliferation of NIH3T3 cells. The strong growth promoting effect of TGF- β 1 can also be observed when cells are cultured in a concentration of 5% new born calf serum, indicating that TGF- β 1 is a potent proliferation-stimulating factor for NIH3T3 and probably other fibroblast cells. The results imply that certain signaling molecules or pathways may be shared or intertwined in both growth stimulation and antiapoptotic events mediated by TGF- β 1.

Figure 5 TGF- β 1 induces the phosphorylation of ERK1 and ERK2 and inhibits serum-deprivation-induced phosphorylation of p38 MAPK. Phosphorylation of ERK1/ERK2 and p38 was examined by immunoblotting as described in the Materials and Methods section. (a) TGF- β 1 induces the phosphorylation of ERK1/ERK2. NIH3T3 cells were incubated in serum-deprived medium for 24 h and treated with TGF- β 1 (5 ng/ml) that was added at different times before the termination of incubation. ERK1/ERK2 and phosphorylated ERK1/ERK2 were examined as described. (b) The effects of serum deprivation on ERK1/ERK2 phosphorylation. Confluent cells (70–80%) were deprived of serum for the times indicated. ERK1/ERK2 phosphorylation was examined as in (a). (c) Serum-deprivation-induced phosphorylation of p38 MAP kinase. Confluent cells (70–80%) were deprived of serum for the times indicated. p38 and phosphorylated p38 MAP kinases were determined as described in the Materials and Methods section. (d) TGF- β 1 suppresses the serum-deprivation-induced phosphorylation of p38 MAP kinase. 70–80% confluent cells were deprived of serum and further incubated for 24 h in the absence or presence of TGF- β 1 (5 ng/ml) that was added at the indicated times before the termination of incubation. p38 and phosphorylated p38 were analyzed by immunoblotting. Ctr (control) represent cells cultured in a medium containing 5% NCS. Data represent one of the four experiments with same results

It has been reported that Erk2 and c-Jun/JNK are involved in TGF- β 1-mediated suppression of serum-deprivation-induced apoptosis in macrophages¹⁶ and in A-459 cells,¹⁷

Figure 6 Effect of transfection of p38 MAP kinase on cell apoptosis. Cells were transfected with vector (pcDNA3, VT) plasmid, wild-type p38 (WT), or kinase-dead mutant p38(AF) within which the T¹⁸⁰ and Y¹⁸² were changed to A and F. Transfection was performed as described in the Materials and Methods section. (a) The expression of wild-type and mutant p38 MAP kinases in NIH3T3 cells was determined 24 h after transfection. (b) After transfection cells were further incubated in regular or serum-deprived medium in the presence or absence of TGF- β 1 (5 ng/ml) for 24 h. Apoptosis determined by FACS assay

respectively. ERK inactivation has been shown to be involved in the sensitization of NIH3T3 cells to undergo apoptosis.³⁰ Ceramide has been shown to function as a progression factor for rho-induced apoptosis in NIH3T3 cells.³¹ In HL-60 cells, TGF- β 1 has been shown to attenuate the ceramide-induced CPP32/Yama protease activation and apoptosis.³² We observed that serum withdrawal induces a progressive apoptotic response of cells that is correlated with the gradual accumulation of intracellular ceramide. Moreover, TGF- β 1's antiapoptotic effect is associated with its inhibition on serum-deprivation-induced increased ceramide formation. The re-

Figure 7 Effect of MAP kinase inhibitors and TGF- β 1 on cell apoptosis and ceramide formation. Confluent NIH3T3 cells (70–80%) cultured in 60-mm plates were incubated in serum-deprived medium for 24 h in the presence or absence of 10 μ M SB203580 (a), 10 μ M PD98059 (b), or 5 ng/ml TGF- β 1. Control (lane 1) cells were cultured in 5% NCS-containing medium. DNA fragmentation in NIH3T3 cells was determined as described in the Materials and Methods section. AO/EB staining assay (c) and FACS assay (d) show the effect of PD98059 (10 μ M), SB203580 (10 μ M), and/or TGF- β 1 (5 ng/ml) on the cell apoptosis. The figure represents one of at least four experiments with the same result. (e) Effect of MAP kinase inhibitors on ceramide formation. Confluent NIH3T3 cells (70%) were labeled with [³H]-serine overnight in normal growth medium, then incubated in fresh medium with or without 5% NCS, and treated with TGF- β 1 (5 ng/ml) for 24 h in the presence of PD98059 (10 μ M) and SB203580 (10 μ M). Ceramide level was determined as described in the Materials and Methods section. The cpm of ceramide from nontreated control cells incubated in 5% NCS is 167.4. Data are the means $\pm$ S.D. of four experiments performed in duplicates

sults demonstrated that inhibition of serum-deprivation-induced ceramide generation is a signaling event that is associated with the antiapoptotic function of TGF- β 1. Since

exogenous cell permeable ceramide analog C₈-ceramide-induced cell apoptosis was not inhibited by TGF- β 1, it suggests that the antiapoptotic effect of TGF- β 1 partly

Figure 8 Effect of MAP kinase inhibitors on cell proliferation. Cells cultured in 24-well plates were incubated in fresh medium without serum and treated with or without TGF- β 1 (5 ng/ml) for 24 h in the presence and absence of PD98059 (a) or p38 MAPK inhibitor SB203580 (b). Cells were labeled with [3 H]-thymidine (0.3 μ Ci/ml), 3 h before the termination of incubation, and the DNA synthesis was determined as described in Materials and Methods section. The cpm of controls for (a) and (b) was 150.6 and 167.4, respectively. Data are the means $\pm$ S.D. of four experiments performed in quadruplicates

contributed to its inhibitory effect on ceramide production but not to its effect on the function of ceramide.

Treatment of serum-deprived NIH3T3 cells with TGF- β 1 induces a gradual and sustained activation of both ERK1 and ERK2, as measured by increased phosphorylated forms of ERK1/ERK2, which are correlated with the suppression of apoptotic response. Inhibition of TGF- β 1-induced activation of ERK1/ERK2 by PD98059 partly blocked the antiapoptotic effect of TGF- β 1, the data indicated that ERK1/ERK2 function as survival signals whose activation are implicated in TGF- β 1-mediated antiapoptotic events. Deprivation of serum in culture medium also induces a rapid and constant activation of p38 MAP kinase as shown by increased phosphorylation of p38. The data present indicate that p38 and ERK1/ERK2 play important but antagonistic roles in serum-deprivation-induced apoptosis. In the presence of TGF- β 1, serum-deprivation-induced activation of p38 was completely inhibited with a corresponding suppression of the cell apoptosis. The evidence indicated that the regulation of p38 MAP kinase is an early and sustained signaling event involved in the serum-

deprivation-induced apoptosis and in the TGF- β 1-mediated antiapoptotic effect in NIH3T3 cells. The finding that inhibition of p38 by the selective inhibitor SB203580 not only significantly suppressed the serum-deprivation-induced apoptosis but also evidently strengthened the antiapoptotic effect of TGF- β 1 provided further support on the role of p38 in serum-deprivation-induced apoptosis. Evidence obtained from expression of wild-type and mutant p38 MAP kinase provided additional support on the role of p38 in NIH3T3 cell apoptosis. Although PD98059 partly inhibits the antiapoptotic function of TGF- β 1 and can moderately increase the intracellular ceramide level, it cannot block the TGF- β 1-mediated suppression of serum-deprivation-induced increase in ceramide formation. These results suggest that ceramide formation is a signaling event upstream the p38 MAP kinase. The increased ceramide production is unlikely a consequence of apoptosis but rather one of the complex signaling components that leads to apoptosis. The evidence may also suggest that ceramide formation is an independent pathway that plays a role in serum-deprivation-induced apoptosis and in the TGF- β 1-mediated antiapoptotic function. The present study indicates that both serum deprivation-induced apoptosis and the TGF- β 1's antiapoptotic function may involve diverse signaling molecules, including ceramide, p38, and ERK1/ERK2.

We have previously demonstrated that TGF- β 1 induces the apoptosis of AML-12 hepatocytes via a mechanism involving the activation of p38 MAP kinase,³³ which was further confirmed and investigated in more detail by our recent study (data not shown). It has been reported that exogenous permeable ceramide analogs C₂-ceramide and C₈-ceramide induce the p38 phosphorylation. Ceramide-induced apoptosis in cortical neurons was believed to be mediated by increased p38 phosphorylation.³⁴ We find that ceramide is able to induce both p38 and ERK phosphorylation in A-431 cells. In NIH3T3 cells, while ceramide has a stimulatory effect on the phosphorylation of ERK, it does not increase the p38 phosphorylation (data not shown). Serum-deprivation-induced p38 phosphorylation appears to be earlier than increased ceramide production. Inhibition of p38 by SB203580 cannot inhibit the ceramide-induced cell apoptosis. Thus, ceramide production and p38 activation are likely to be parallel or independent events. It is interesting that both stimulatory and inhibitory effect of TGF- β 1 on cell apoptosis was mediated via a mechanism involving p38 and ERK1/ERK2 MAP kinases. The antagonistic effects produced by TGF- β 1 were thus at least partly resulted from their opposite regulation, that is, the inhibition and activation of p38 and ERK1/ERK2 MAP kinases. In addition, inhibition of increased ceramide generation may account partly the functions of TGF- β 1 in the suppression of cell apoptosis. While treatment of cells with PD98059 completely suppressed the TGF- β 1-stimulated cell proliferation, the inhibition of PD98059 on TGF- β 1-mediated suppression of apoptotic cell death was relatively much less potent. Conversely, SB203580 showed no effect on the basal level of cell proliferation, it significantly enhanced the TGF- β 1-stimulated cell proliferation. It therefore indicates that p38 functions mainly as an apoptotic factor, whereas ERK1/ERK2 may function primarily as surviving and proliferation-stimulating factors that play important roles in the TGF- β 1-mediated proliferation and antiapoptotic events. The

presented evidence also suggests that TGF- β 1-mediated cell proliferation and antiapoptosis may be inseparable events involving the same signaling components.

The fact that TGF- β 1 is able to cause antagonistic effect (induction or suppression) in cell proliferation, differentiation, and apoptosis contains a contradictory or mutually exclusive condition. The living body does not abolish this contradiction during its life course, but develops this contradiction and the form within which this contradiction is able to move. The reverse stimulation or inhibition of p38 and ERK1/ERK2 MAP kinases and probably the ceramide production by TGF- β 1 constitute a solution for the opposing effects of TGF- β 1 in apoptosis and in proliferation (Figure 9). It has been reported recently that the opposite effects of TGF- β on endothelium were mediated by two distinct TGF- β type I receptors. TGF- β /ALK5 pathway inhibits the cell migration and proliferation, but the TGF- β /ALK1 pathway induces endothelial cell migration and proliferation.³⁵ Since the diverse effects of TGF- β depend on the cell types and their various environments, further study on signaling basis by which different types of cells are differentially regulated by TGF- β may be helpful in deepening our understanding on some physiological and pathological processes.

Materials and Methods

Materials

Cell culture reagents and fetal bovine serum (FCS) were purchased from Gibco BRL. EN³HANCETM Spray, [³H]-serine (28 Ci/mmol), [³²P- γ]-ATP (3000 Ci/mmol), [*N*-methyl-¹⁴C]-SM (55 mCi/mmol), BCS (Biodegradable Counting Scintillant) scintillation cocktail, and nitrocellulose membrane were bought from Amersham Pharmacia Biotech (Buckinghamshire, UK). [³H]-thymidine (81 Ci/mmol) was from NEN Life Science Products (Boston, MA, USA). Ceramide standard was bought from Avanti Polar Lipids (Alabaster, AL, USA). SMase, NOE, PD98059, SB203580, C₆-ceramide, Caspase inhibitor III, Caspase-1 substrate IV, Caspase-1 substrate VII, Caspase-3 substrate I, Caspase-6 substrate II, and Caspase-8 substrate I were purchased from Calbiochem (San Diego, CA, USA). Silica gel 60 Å thin layer chromatography (TLC) plates were purchased from Whatman (Clifton, NJ, USA). Enhanced chemiluminescent reagents (ECL), antibodies against ERK2, p38 and phosphorylated ERK1/2, horseradish peroxidase (HRP)-conjugated anti-mouse, and anti-rabbit antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibody against phosphorylated p38 was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against FLAG was from Sigma (St Louis, MO, USA).

Cell culture

NIH3T3 fibroblast cells (originally from ATTC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% (v/v) FCS (Gibco BRL), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco BRL) and maintained in a humidified atmosphere and 5% CO₂ at 37°C. Medium was renewed every 2–3 days until confluence was reached. Serum-deprived culture medium containing 0.5% BSA was used to make cells quiescent or to induce cell apoptosis. For determination of cell apoptosis, cells were seeded into 60-mm plates and cultured under the same condition until confluence was reached.

Figure 9 Schematic presentation on the regulation of serum-deprivation-induced apoptosis by TGF- β 1. ERK1/ERK2 functions primarily as survival and growth stimulation factors, whereas p38 and ceramide function primarily as death-promoting factors. Deprivation of cells from serum induces a gradual but sustained ceramide increase and the continued activation of p38 MAP kinases, which is associated with the inhibition of cell proliferation and the induction of cell apoptosis. TGF- β 1 that activates ERK1/ERK2, inhibits the p38 MAP kinase, and blocks the increased ceramide formation, promotes the cell proliferation, and suppresses the serum-deprivation-induced apoptosis

Labeling of cells

Cells (80% confluence) were incubated in a medium containing 0.5 or 5% NCS (Gibco BRL), and then labeled with indicated radioactive compound for 16 h. [³H]-serine (4 μ Ci/ml) was used to label intracellular ceramide. To label the cells for proliferation assay, [³H]-thymidine (0.3 μ Ci/ml) was added during the last 1 (for cells cultured in 5% NCS) or 3 h (for cells cultured in 0.5% NCS) of incubation.

Cell proliferation assay

Cells were grown in 24-well plates to about 80% confluence, then incubated in 200 μ l of 0.5% NCS or 5% NCS for additional 24 h and labeled with [³H]-thymidine. Indicated reagents and/or inhibitors were added at different time points during the incubation. The incubation was terminated by pouring off the medium and washing the cells with ice-cold phosphate-buffered saline (PBS) 3 times followed by adding 200 μ l of 12% trichloroacetic acid (TCA). The plates were maintained on ice for 30 min and washed again with ice-cold deionized water three times. The acid-insoluble material was redissolved in 200 μ l lysis buffer (1% SDS, 0.2 N NaOH) and moved into scintillation vials, mixed with 2.5 volumes of Biodegradable Counting Scintillant (BCS). The DNA-associated [³H]-radioactivity was determined by liquid-scintillation spectrometry (Wallac 1409, Pharmacia).

Analysis of phosphorylation of ERK and p38 MAP kinases

The phosphorylation of ERK and p38 MAPK was analyzed by immunoblotting as described by Ushio-Fukai *et al.*³⁶ NIH3T3 cells were lysed in 0.4 ml of ice-cold lysis buffer (50 mM HEPES pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 10 mM Na₄P₂O₇ · H₂O, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 1 mM PMSF). The lysates were centrifuged at 12 000 g for 10 min. The supernatants were collected and separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond ECL, Amersham

Life Sciences). The membranes were blocked with 5% nonfat dry milk in tris-buffered saline containing 0.1% Tween-20 (TBS-T) and subsequently incubated with mouse antiphospho-ERK monoclonal antibody (1 : 2000) or rabbit antiphospho-p38 polyclonal antibody (1 : 2000). Antibodies against ERK2 and p38 MAP kinase were used for determining the protein levels of these proteins. Specific protein bands were detected using HRP-conjugated goat anti-mouse or anti-rabbit IgG (1 : 4000) and visualized by ECL reagents.

Measurement of ceramide and SM

Cells were cultured in 60-mm plates to about 70% confluence, then labeled with [3 H]-serine (4 μ Ci/ml) overnight, followed by changing the medium and the treatment of cells with indicated reagents. After further incubation for the times as indicated, the reaction was terminated and the total lipids in the organic phase were extracted and then loaded to TLC plate for analysis as previously described.³⁷ SM was separated by the solvent system of CHCl₃:CH₃OH:acetic acid:H₂O (50:25:4:2; v:v). Ceramide was separated by a solvent system of n-hexane:diethyl ether:methanol:acetic acid (90:20:6:3; v:v). The respective lipids separated were scrapped from TLC plates into scintillation vials. Lipid molecules were eluted out of the gel by adding 500 μ l of methanol and then mixed with 2.5 ml of BCS scintillation cocktail. Total radioactivity incorporated into the lipids of each sample was counted by taking 10 μ l of the organic phase of the lipid extracts. The radioactivity was determined by liquid-scintillation spectrometry. The percentage of total radioactivity for ceramide and SM of each sample was calculated and present as fold increase.

SMase activity assay

SMase activity was measured as previously described.³⁸ Briefly, cells cultured in 60-mm plates were lysed in the following buffers, respectively: (1) neutral Mg²⁺-dependent: 0.1% Triton X-100, 5 mM MgCl₂, and 20 mM HEPES (pH 7.4); (2) neutral Mg²⁺-independent: 0.1% Triton X-100 and 20 mM HEPES (pH 7.4); (3) acidic: 0.1% Triton X-100 and 0.1 M sodium acetate (pH 5.0). The lysates were centrifuged at 12 000 g for 10 min. The supernatants were collected and incubated with 250 μ M cold SM, 0.1 μ Ci/ml [14 C]-SM, and 1 mM ATP in 50 μ l assay buffer (total) at 37°C for 1 h, using 800 μ l CHCl₃:CH₃OH (2:1) and 200 μ l H₂O to terminate the reaction. The water phase was extracted and mixed with 2.5 volumes of biodegradable counting scintillant (BCS). The [14 C]-phosphocholine radioactivity was determined by liquid-scintillation spectrometry.

Acridine orange/ethidium bromide (AO/EB) staining

AO/EB staining for morphologic examination was conducted as described³⁹ with minor modifications. NIH3T3 cells cultured in 35-mm plates were treated as indicated, and stained by adding AO/EB solution directly into the medium (final concentration for each was 2 μ g/ml). The stained cells were observed under a fluorescent microscope, the wide-band blue excitation (wavelength 450–480 nm) was used.

Flow cytometry analysis

Cell apoptotic rate was quantitatively determined by flow cytometry analysis.^{40,41} The percentage of cells with a sub-G₁ DNA content was taken as a measure of the apoptotic rate of the cell population. After indicated treatment, cells were trypsinized and fixed with

70% ethanol for over an hour. Cells were then pelleted and washed with PBS plus 20 mM EDTA. RNA was removed by adding RNase (1 mg/ml) at 37°C for at least 2 h. Finally, the cells were stained with propidium iodide (final concentration: 30 μ g/ml) and analyzed the DNA content by FACS (Becton Dickinson FACS Calibur, the excitation wavelength is 488 nm).

DNA fragmentation assay

Internucleosomal DNA degradation was examined as described by Lindenboim *et al.*⁴² with minor modifications. Briefly, untreated and treated NIH3T3 cells in 60-mm plates were collected and lysed in 0.4 ml of lysis buffer (10 mM Tris pH 7.4, 25 mM EDTA, and 0.25% Triton X-100) on ice for 30 min. After centrifugation at 13 800 g for 15 min, the supernatants were collected and incubated with RNase (200 μ g/ml) at 37°C for at least 1 h and then further incubated with proteinase K (100 μ g/ml) at 56°C overnight. The samples were then extracted sequentially with phenol, phenol-chloroform, and chloroform. DNA in aqueous phase was precipitated by adding 1/10 vol. of 5 M NaCl and 2 vol. of ethanol at –20°C overnight. The DNA was recovered and analyzed by agarose gel (1.2%) electrophoresis (30 V for 3–4 h).

Caspase activity assay

NIH3T3 cells cultured in 60-mm plates were treated as indicated, then trypsinized and washed once with PBS. Cells were collected and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA) for 10 min, and were centrifuged at 12 000 g at 4°C for 10 min. The supernatants were collected and incubated with 400 μ M caspase substrate in 100 μ l caspase activity assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol) for 2 h at 37°C. Optical density (O.D.) was measured at 405 nm.

Cell transfection

The plasmids were transfected into the cells according to the manufacturer's instructions. Briefly, 2 μ g plasmid DNA in 50 μ l DMEM and 10 μ l lipofectamine in 50 μ l DMEM were mixed gently and incubated at room temperature for 30 min. The cells were trypsinized and mixed with the DNA-lipofectamine complex. The mixtures were rotated with end-over-end mixing for about 10 min at room temperature with high speed. Cells were equally divided in culture plates and incubated in 10% FCS containing DMEM (without penicillin and streptomycin). After 24 h, the medium was replaced with fresh normal DMEM culture medium. The expression of p38 was identified by immunoblotting.

Statistical analysis

Results are presented as means $\pm$ standard deviations (s.d.) for the number of experiments indicated. For statistical analysis, Student's *t*-test was used. Differences were considered significant at a level of *P* < 0.05.

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