Transforming growth factor β 1 attenuates ceramide-induced CPP32/Yama activation and apoptosis in human leukaemic HL-60 cells

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Ceramide, a product of sphingomyelin turnover, is a novel lipid second messenger that mediates important cellular functions including proliferation, differentiation and apoptosis. This study demonstrates that the CPP32/Yama protease was activated during apoptosis induced by the membrane-permeable second messenger C_2 -ceramide in HL-60 cells. We also found that the addition of a specific tetrapeptide inhibitor of CPP32/Yama, Ac-DEVD-CHO, provided an effective protection against ceramide-induced cell death. These results suggested that CPP32/Yama has a central role in ceramide-mediated apoptosis. Furthermore a wide variety of cytokines were examined for their effect on ceramide-induced apoptosis. Only transforming growth factor β 1 (TGF- β 1) (1 ng/ml) exerted significant prevention of apop-

tosis induced by C_2 -ceramide, or by sphingomyelinase (increases intracellular ceramide). Consistently, TGF- β 1 abrogated the cleavage of poly(ADP-ribose) polymerase and the production of the CPP32/Yama active subunit, p17. However, TGF- β 1 treatment did not cause growth inhibition or alter the level of cyclin-dependent kinase inhibitor p27. It suggests that the preventive effect of TGF- β 1 is not mediated by growth arrest. Interestingly, we found that TGF- β 1 prevented the C_2 -ceramide-caused decrease of Bcl-2 protein. We thus propose that TGF- β 1 rescues ceramide-induced cell death, possibly by maintaining the constant level of Bcl-2, thereby abolishing CPP32/Yama protease activation.

INTRODUCTION

Ceramide, a product of sphingomyelin hydrolysis, is an important mediator of a number of extracellular stimuli including, tumour necrosis factor α (TNF α) [1], interferon (IFN) γ [2], interleukin (IL) 1 [3], 1,25-dihydroxyvitamin D₃ [4] and chemotherapeutic agents [5,6]. Ceramide can induce cell differentiation [7], growth inhibition [8] or apoptosis [9] in various cell systems. The mechanism by which ceramide induces such cellular effects is poorly understood. Intense interest has focused on the potential involvement of diverse signalling processes in the regulation of ceramide-mediated apoptosis. Sawai et al. revealed that c-jun/AP-1 is activated by ceramide in the early stages of apoptosis and that the impairment of AP-1 activity by anti-sense oligonucleotides rescues apoptosis [10]. Verheij et al. [11] support this finding by showing that the activation of Jnk, a c-Jun N-terminal kinase, was required for ceramide-initiated apoptosis. Furthermore ceramide suppresses *bcl-2* mRNA production in apoptotic cells [12]. Ceramide might thus modulate Bcl-2 gene expression to facilitate cell death. Ectopic overexpression of *bcl-2* results in an almost total protection of cell death in response to ceramide [13]. Recent work has indicated the critical involvement of CPP32/Yama protease in cell death programmed by events such as Fas ligation or TNF α receptor activation [14,15]. Emerging evidence has suggested that *bcl-2* expression interrupts the proteolytic activation of CPP32/Yama, thereby preventing cell death [16,17]. The anti-apoptotic protein Bcl-2 might thus act at a point upstream of CPP32/Yama. It is known that ceramide induces apoptosis by modulating Bcl-2 expression. However, the effect of the second messenger on the CPP32/Yama protease is unclear.

The growth, differentiation and apoptosis of haemopoietic cells depend on a complex interplay between progenitor cells and other cells residing in the bone marrow microenvironment.

Many soluble factors (for example cytokines) secreted by these cells have key roles in normal and malignant haemopoietic cell death and differentiation [18]. Colony-stimulating factors and interleukins, for example, are regulators promoting cell survival [19,20]; transforming growth factor β 1 (TGF- β 1) and TNF α are regulators initiating cell death [21,22]. However, the critical point determining the fate of cellular response to these cytokines is cell-type-dependent [23]. The present study shows that cysteine protease CPP32/Yama participates in ceramide-induced apoptosis in human leukaemic HL-60 cells. TGF- β 1, but not other cytokines, effectively inhibits ceramide-induced cell death by maintaining Bcl-2 levels, thereby inhibiting CPP32/Yama activation.

MATERIALS AND METHODS

Cell culture and chemicals

HL-60 (a human promyelocytic leukaemia cell line), U937 (a human monocytic leukaemia cell line) and MOLT-4 (human T-cell leukaemia), purchased from American Type Culture Collection (Rockville, MD, U.S.A.), were cultured in RPMI 1640 supplemented with 10% (v/v) fetal calf serum. C_2 -ceramide, sphingomyelinase, propidium iodide and Proteinase K were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). TGF- β 1 and granulocyte/macrophage colony-stimulating factor (GM-CSF) were purchased from R & D Systems (Minneapolis, MN, U.S.A.). IFN γ , IL-3 and IL-6 were generally provided by Dr. J. Y. Yen (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan).

DNA fragmentation assay

Cells were harvested, washed with PBS and DNA fragmentation was analysed as described previously [24].

Abbreviations used: PARP, poly(ADP-ribose) polymerase; TGF- β 1, transforming growth factor β 1; TNF α , tumour necrosis factor α ; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; IFN, interferon.

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Quantification of apoptosis by flow cytometry

Cells used for cytometry were prepared as described previously [24]. Briefly, 10^6 cells were washed with PBS and resuspended in 500 μ l of a buffer (0.5% Triton X-100/PBS/0.05% RNase A) and incubated for 30 min. Finally, 0.5 ml of propidium iodide solution (50 μ g/ml) was added, and cells were left on ice for 15–30 min. Fluorescence emitted from the propidium iodide–DNA complex was quantified after laser excitation of the fluorescent dye by FACSsor flow cytometry (Becton Dickinson). The extent of apoptosis was determined by counting cells of DNA content below the G_0/G_1 peak.

Immunoblotting

Cellular lysates were prepared as described previously [25]. A 50 μ g sample of each lysate was subjected to SDS/PAGE [10% (w/v) gel] for detection of Bcl-2, poly(ADP-ribose) polymerase (PARP), CPP32/Yama and p27^{kip}. The samples were then electroblotted on nitrocellulose paper. After blocking, blots were incubated with anti-(Bcl-2), anti-PARP, anti-p27 or anti-CPP32 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) antibodies in PBST (0.1% Tween-20/PBS) for 1 h followed by two washes (15 min each) in PBST, and then incubated with horseradish peroxidase-conjugated goat anti-(mouse IgG) or anti-(rabbit IgG) (Amersham, Des Plaines, IL, U.S.A.) for 30 min. After washing, blots were incubated for 1 min with Western blotting reagent enhanced chemiluminescence (Amersham); chemiluminescence was detected by exposure of the filters to Kodak X-Omat films for between 30 s and 30 min.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. for three separate experiments. The significance of difference was assessed by using one-way analysis of variance (ANOVA) followed by the Student's *t* test.

RESULTS

We intended to determine whether CPP32/Yama participates in the ceramide-mediated apoptotic process. To address this issue we examined the cleavage of PARP protein and CPP32/Yama by using Western blotting. When human promyelocytic leukaemic HL-60 cells were treated with the lipid second messenger C_2 -ceramide at 25 μ M, cleavage of PARP protein was initially detected 4 h after exposure (Figure 1a, lane 3). The anti-PARP antibodies used here recognize an epitope at the N-terminus of PARP polypeptide; the N-terminal 31 kDa fragment can thus be detected by immunoblotting. The proteolytic cleavage of pro-CPP32/Yama to produce two subunits, p17 and p12, is essential for the formation of active CPP32 protease. In parallel with PARP cleavage, the appearance of active p17 CPP32 subunits was also observed 4 h after C_2 -ceramide treatment (Figure 1b, lane 3). The level of the p17 active subunits gradually increased with time. However, the level of cytoskeleton component α -tubulin protein remained constant during exposure to the agent (Figure 1c), indicating that the degradation of PARP protein and of CPP32 is a specific proteolytic process in response to ceramide. Under similar conditions, the formation of internucleosomal DNA fragments, a hallmark of apoptosis, was first observed 4 h after ceramide treatment (Figure 1d). We therefore further pretreated cells with 200 μ M specific tetrapeptide CPP32 inhibitor, Ac-DEVD-CHO, for 1 h, and examined its effect on ceramide-mediated PARP cleavage as well as DNA fragmentation. The addition of tetrapeptide inhibitor Ac-DEVD-CHO

Figure 1 The kinetics of C_2 -ceramide-induced PARP cleavage, CPP32/Yama activation and DNA fragmentation in human leukaemia HL-60 cells

Western analyses of PARP cleavage (a), CPP32/Yama proteolytic activation (b) and α -tubulin protein (c) in HL-60 cells treated with 25 μ M for the indicated times. Samples were subjected to electrophoresis and the intact proteins and fragments were detected by immunoblotting as described in the Materials and methods section (d) Agarose gel electrophoresis of 25 μ M C_2 -ceramide-induced DNA fragmentation. Abbreviation: K, kDa.

evidently prevented DNA fragmentation (Figure 2b) and PARP cleavage (Figure 2a). These results strongly suggested that activation of CPP32/Yama protease is necessary in C_2 -ceramide-mediated apoptosis.

Next we examined whether ceramide-induced cell death would be affected by some haemopoietic cytokines. The extent of apoptosis was quantified by counting cells that possessed a DNA content below the G_0/G_1 peak (sub- G_1 fraction) by using flow cytometry. Preincubation of cells with cytokines such as IL-6 (10 ng/ml), IL-3 (10 ng/ml), IFN- γ (1000 i.u./ml), and GM-CSF (5 and 10 ng/ml) for 1 h exerted no effect on C_2 -ceramide-induced apoptotic death (Table 1). Interestingly, pretreatment with as low a concentration of TGF- β 1 as 1 ng/ml for 1 h significantly protected cells from C_2 -ceramide-mediated cell death. TGF- β 1 also inhibited sphingomyelinase (56 m-units/ml)-induced apoptosis. An increment in TGF- β 1 concentration up to 10 ng/ml provided similar protection against cell death caused by C_2 -ceramide (results not shown), implying that the protective action of TGF- β 1 might be mediated through binding of the cytokine to its receptor. We further examined the inhibitory activity of TGF- β 1 on other ceramide-induced cellular changes such as DNA laddering and morphological alteration. As expected, TGF- β 1 effectively abrogated ceramide or sphingomyelinase-induced DNA fragmentation (Figure 3a) and the formation of apoptotic bodies (Figure 3b). U937 and Molt-4 cells also underwent apoptosis in response to C_2 -ceramide. However, TGF- β 1 inhibited the apoptosis induced in these cell

C2-Ceramide (μ M) - 25 25 -
 DEVD-CHO (μ M) - - 200 200

Figure 2 Inhibition of (a) the PARP cleavage activity and (b) DNA fragmentation of C₂-ceramide-treated HL-60 cells by CPP32 tetrapeptide inhibitor

HL-60 cells were incubated with 25 μ M C₂-ceramide in the absence or presence of 200 μ M Ac-DEVD-CHO, a specific inhibitor of CPP32/Yama, for 6 h; cells were then divided into two portions: one was subjected to Western blot analysis, the other to agarose-gel analysis of DNA fragmentation. Abbreviation: K, kDa.

Table 1 Effects of various cytokines on cell death induced by ceramide

HL-60 cells (5×10^5) were treated for 24 h with various combinations of agents as indicated. Cells with apoptotic DNA were measured by sub-G₁ analysis (see the text). Values shown are means $\pm$ S.E.M. for three separate experiments. * Statistically significant decrease in apoptosis compared with ceramide-treated or sphingomyelinase-treated group ($P < 0.05$).

Treatment	Concentration	Apoptosis (%)
None		7.1 $\pm$ 1.7
C2-ceramide	25 μ M	56.8 $\pm$ 4
+ TGF- β 1	1 ng/ml	25.1 $\pm$ 3.8*
+ TGF- β 1	5 ng/ml	24.7 $\pm$ 4.9*
+ IL-3	10 ng/ml	55.6 $\pm$ 3.3
+ IL-6	10 ng/ml	54.2 $\pm$ 2.9
+ IFN- γ	1000 i.u./ml	62.2 $\pm$ 5.2
+ GM-CSF	5 ng/ml	53.2 $\pm$ 6.2
+ GM-CSF	10 ng/ml	52.1 $\pm$ 3.1
Sphingomyelinase	56 m-units/ml	45.6 $\pm$ 4.5
+ TGF- β 1	1 ng/ml	25.3 $\pm$ 6.0*
+ TGF- β 1	5 ng/ml	25.9 $\pm$ 4.4*
+ GM-CSF	10 ng/ml	46.5 $\pm$ 3.3
+ IL-3	10 ng/ml	44.4 $\pm$ 2.9
TGF- β 1	5 ng/ml	7.2 $\pm$ 4.4
GM-CSF	10 ng/ml	6.2 $\pm$ 4.0
IL-3	10 ng/ml	6.5 $\pm$ 2.6
IL-6	10 ng/ml	5.8 $\pm$ 4.3
IFN- γ	1000 i.u./ml	5.5 $\pm$ 4.1

lines to a lesser extent (Figure 3c). The differential activity suggests that different effects of TGF- β 1 on the ceramide-mediated cell-death signal transduction pathway exist in different haemopoietic cellular contexts.

Figure 3 Effect of TGF- β 1 on ceramide-induced DNA fragmentation and apoptotic morphology

(a) Agarose gel analysis of chromosomal DNA from HL-60 cells treated with different agents for 6 h as indicated. Abbreviation: SMase, sphingomyelinase. (b) Microscopic examination of HL-60 cells treated with: panel I, solvent control [0.1% (v/v) DMSO]; panel II, 25 μ M C₂-ceramide; panel III, 1 ng/ml TGF- β 1 plus 25 μ M C₂-ceramide; panel IV, 1 ng/ml TGF- β 1. Magnification $\times$ 60. (c) Agarose-gel analysis of chromosomal DNA from U937 and Molt-4 cells treated with different agents for 9 h as indicated.

Figure 4 Western blot analysis of TGF- β 1 effects on C₂-ceramide-mediated PARP cleavage (a) and CPP32/Yama activation in HL-60 cells (b)

Cells, with or without a 1 h pretreatment with 1 ng/ml TGF- β 1, were exposed to 25 μ M C₂-ceramide for 10 h. Abbreviation: K, kDa.

Figure 5 Effects of TGF- β 1 on the level of cyclin-dependent kinase inhibitor p27 by Western blot analysis

HL-60 cells were treated with 1 ng/ml TGF- β 1 or 1 ng/ml TGF- β 1 plus 25 μ M C₂-ceramide for the indicated times. Samples were subjected to electrophoresis and the p27 protein was detected as described in the Materials and methods section.

CPP32 cysteine protease plays a central role in regulating ceramide-mediated apoptosis (Figures 2a and 2b). We therefore asked whether TGF- β 1 affects ceramide-induced CPP32 activation and PARP cleavage. We found that the cleaved 31 kDa PARP fragment, formed in response to ceramide treatment, disappeared in the presence of TGF- β 1 (Figure 4a, compare lanes 2 and 3). The inhibitory ability of TGF- β 1 on PARP cleavage was comparable to that of CPP32 tetrapeptide inhibitor, Ac-DEVD-CHO (compare Figure 4a, lane 3, with Figure 2a, lane 3). Next we examined the effect of TGF- β 1 on CPP32 activation by ceramide. Again, the concentration of the active subunit p17 of CPP32 induced by ceramide was effectively decreased by TGF- β 1 (Figure 4b). Taken together, these results suggest that the pluripotent cytokine TGF- β 1 has a counteractive role in ceramide-induced apoptotic cell death in some haemopoietic cells.

TGF- β 1 induces cell growth arrest through the activation of cyclin-dependent kinase inhibitor p27 [26,27]. We wondered whether TGF- β 1 prevented ceramide-mediated apoptosis through its growth inhibitory effect. To address this we examined whether TGF- β 1 affected the cellular growth of HL-60 cells as determined by a [³H]thymidine incorporation assay. Although TGF- β 1 at the concentration of 1 ng/ml effectively prevented ceramide-induced cell death, it did not inhibit HL-60 cell growth

Figure 6 Effects of TGF- β 1 on C₂-ceramide-induced changes in Bcl-2 level by Western blot analysis

(a) Inhibition of C₂-ceramide-induced depletion of Bcl-2 by TGF- β 1. (b) Effect of TGF- β 1 on the level of Bcl-2. Cells, with or without a 1 h pretreatment with 1 ng/ml TGF- β 1, were exposed to 25 μ M C₂-ceramide for 10 h.

(results not shown). A Western blot shows that TGF- β 1 or TGF- β 1 plus ceramide did not change the level of p27 in HL-60 cells (Figure 5).

Treatment of HL-60 cells with ceramide resulted in a decrease of *bcl-2* mRNA and ultimately led to cell death [12]. We therefore considered the possibility that the protection of TGF- β 1 against ceramide-induced apoptosis operated at the level of *bcl-2*. Consistent with previous reports by others [12], we found that the level of Bcl-2 protein was decreased on treatment with ceramide (Figure 6a, lane 2). The decrease could be completely rescued by TGF- β 1 (Figure 6a, lane 3). However, treatment of cells with TGF- β 1 alone did not change the level of Bcl-2 (Figure 6b).

DISCUSSION

We have demonstrated here that CPP32/Yama peptide inhibitor, Ac-DEVD-CHO, effectively abrogated C₂-ceramide-induced DNA fragmentation, PARP cleavage and apoptotic morphology. This provides the insight that CPP32/Yama has a prominent role in ceramide-mediated apoptotic cell death. Similar findings have recently been reported by others [28,29]. Although a number of stimuli, including Fas, TNF- α and chemotherapeutic agents, induce ceramide production and cause cell death, it seemed that only Fas-induced cell death can be rescued by CPP32 tetrapeptide inhibitor [15]. These observations suggest an indispensable role for ceramide in the signal transduction of Fas-eliciting apoptotic cell death.

TGF- β 1, a multifunctional cytokine, is produced by a variety of immunocompetent cells [30] and can act either to stimulate or to inhibit cellular proliferation, cellular differentiation or other cellular functions [31]. TGF- β 1 triggered apoptosis in a wide variety of cell types including hepatocytes [32], hepatocellular carcinoma [33], B-cell lymphoma [34] and M1 leukaemia [35]. In contrast, it was particularly striking to demonstrate the unexpected ability of TGF- β 1 to block ceramide-mediated CPP32/Yama activation and apoptosis. A similar finding by Cerwenka et al. [36] has shown that TGF- β 1 counteracted Fas-induced apoptosis in human T lymphocytes. Furthermore, Zhang et al. [37] demonstrated that TGF- β 1 and IL-2 synergized to block antigen-stimulated apoptosis almost completely, resulting in prolonged effector expansion and leading to the accumulation

of a large pool of specific effectors. TGF- $\beta 1$ was found to inhibit Fas antigen-mediated apoptosis in rheumatoid synovial cells, resulting in the perpetuation of synovial hyperplasia in patients with rheumatoid arthritis [38]. Combining these data, we suggest that the effects of TGF- $\beta 1$ on apoptosis seem to depend on the type of cell, its state of differentiation or growth and the presence or absence of other regulatory factors. Moreover, a specific and potential role of TGF- $\beta 1$ in antagonizing the Fas pathway is evident but the mechanism is not clear.

C₂-ceramide binds to serum proteins in the medium; this binding might therefore decrease its cytotoxicity [39]. The preventive action of TGF- $\beta 1$ might therefore simply be due to non-specific binding with C₂-ceramide out of the cells. To exclude the possibility we treated cells with sphingomyelinase, an enzyme that generates intracellular ceramide, and found that TGF- $\beta 1$ also rescued sphingomyelinase-induced cell death. Furthermore, because a higher concentration of TGF- $\beta 1$ (10 ng/ml) provided no further protection than did a low concentration (1 ng/ml), a receptor-mediated function of TGF- $\beta 1$ was implicated. However, it is difficult at present to find direct evidence of receptor binding owing to the lack of a specific antibody to block the TGF- $\beta 1$ receptor of HL-60 cells (type 1 and 2).

How does TGF- $\beta 1$ protect cells from ceramide-induced death? Treatment with 1 ng/ml TGF- $\beta 1$ did not affect DNA synthesis (results not shown) or the expression of p27^{kip} (Figure 5) in HL-60 cells, indicating that the TGF- $\beta 1$ -prevented ceramide-mediated cell death was not due to growth arrest. Ectopic overexpression of *bcl-2* in cells confers resistance to ceramide-induced cell death [13]. Furthermore the overexpression of *bcl-2* prevents CPP32 cleavage in apoptotic cells [40], possibly by preventing some steps leading to the proteolytic cleavage of proCPP32 protease. Although it is theoretically possible that Bcl-2 acts directly in preventing the proteolytic cleavage of proCPP32 protease, it seems more likely that Bcl-2 exerts its blocking function upstream of this proteolytic activation event, because the subcellular localization of Bcl-2 seems to differ from that of CPP32 [41]. We found that treatment of cells with ceramide resulted in an inhibition of *bcl-2* expression at both the mRNA and protein levels, which is accordant with an increase in CPP32 cleavage products. This suggests that the depletion of Bcl-2 protein and the subsequent activation of CPP32 proteolytic activity are probably the major causes of ceramide-mediated cell death. TGF- $\beta 1$ seems to be a potent inhibitor of the proteolytic activation of CPP32, possibly through the maintenance of the level of *bcl-2* mRNA and protein. However, the mechanism is unclear.

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