Apoptosis inhibitory activity of cytoplasmic p21^{Cip1/WAF1} in monocytic differentiation

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p21^{Cip1/WAF1} inhibits cell-cycle progression by binding to G₁ cyclin/CDK complexes and proliferating cell nuclear antigen (PCNA) through its N- and C-terminal domains, respectively. The cell-cycle inhibitory activity of p21^{Cip1/WAF1} is correlated with its nuclear localization. Here, we report a novel cytoplasmic localization of p21^{Cip1/WAF1} in peripheral blood monocytes (PBMs) and in U937 cells undergoing monocytic differentiation by *in vitro* treatment with vitamin D3 or ectopic expression of p21^{Cip1/WAF1}, and analyze the biological consequences of this cytoplasmic expression. U937 cells which exhibit nuclear p21^{Cip1/WAF1} demonstrated G₁ cell-cycle arrest and subsequently differentiated into monocytes. The latter event was associated with a cytoplasmic expression of nuclear p21^{Cip1/WAF1}, concomitantly with a resistance to various apoptogenic stimuli. Biochemical analysis showed that cytoplasmic p21^{Cip1/WAF1} forms a complex with the apoptosis signal-regulating kinase 1 (ASK1) and inhibits stress-activated MAP kinase cascade. Expression of a deletion mutant of p21^{Cip1/WAF1} lacking the nuclear localization signal (Δ NLS-p21) did not induce cell cycle arrest nor monocytic differentiation, but led to an apoptosis-resistant phenotype, mediated by binding to and inhibition of the stress-activated ASK1 activity. Thus, cytoplasmic p21^{Cip1/WAF1} itself acted as an inhibitor of apoptosis. Our findings highlight the different functional roles of p21^{Cip1/WAF1}, which are determined by its intracellular distribution and are dependent on the stage of differentiation.

Keywords: apoptosis/cell differentiation/cytoplasmic p21/nuclear p21

Introduction

The p21^{Cip1/WAF1} gene was identified through the interaction of its product with cyclin-dependent kinase (CDK)

Cdk2 (Harper *et al.*, 1993), and by being a gene whose expression is induced by activation of wild-type p53 (El-Deiry *et al.*, 1993) or during cellular senescence (Noda *et al.*, 1994). p21^{Cip1/WAF1} inhibits cell cycle progression by binding to G₁ cyclin-CDK complexes through its N-terminal domain. The gene product also binds proliferating cell nuclear antigen (PCNA) through its C-terminal domain and blocks the ability of PCNA to activate DNA polymerase δ , the principal replicative DNA polymerase (Waga *et al.*, 1994; Chen *et al.*, 1995; Luo *et al.*, 1995; Sherr and Roberts, 1995). In normal fibroblasts, these cell cycle regulators form quaternary complexes consisting of p21^{Cip1/WAF1}, PCNA, cyclin and CDK (Li *et al.*, 1994; Zhang *et al.*, 1994). In addition, recent studies have shown that p21^{Cip1/WAF1} promotes the association of Cdk4 with D-type cyclins, and targets Cdk4 and cyclin D1 to the nucleus (LaBaer *et al.*, 1997) by its bipartite nuclear translocation signal. Thus, the cell cycle inhibitory activity of p21^{Cip1/WAF1} is intimately correlated with its nuclear localization and this property appears to be responsible for the early stages of the differentiation program (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Halevy *et al.*, 1995; Andres and Walsh, 1996).

Recently, another important role for p21^{Cip1/WAF1} in the protection of cells against apoptosis has been proposed. Accordingly, increased susceptibility to p53-mediated apoptosis in p21^{Cip1/WAF1}-deficient cells was observed in colorectal carcinomas (Polyak *et al.*, 1996) and melanomas (Gorospe *et al.*, 1997). Inhibition of stress-mediated apoptosis in MCF-7 cells treated with prostaglandin A2 is also associated with p21^{Cip1/WAF1} expression (Guadagno and Newport, 1996). Together, these recent findings indicate that p21^{Cip1/WAF1} plays a fundamental role in the protection against cytotoxic stimulation of certain cell types. However, the mechanism by which p21^{Cip1/WAF1} antagonizes apoptosis is still unknown.

In this study, we show that the function of p21^{Cip1/WAF1} as an inhibitor of cell cycle progression or of apoptosis is determined by its subcellular localization. p21^{Cip1/WAF1} ectopically expressed in immature monocytes is localized in the nucleus and induces cell cycle arrest associated with differentiation induction. The differentiation of immature monocytes is associated with a relocation of nuclear p21^{Cip1/WAF1} to the cytoplasm. Cytoplasmic p21^{Cip1/WAF1} forms a physical complex with apoptosis signal-regulating kinase 1 (ASK1) and inhibits activation of stress-activated ASK1 and SAPK/JNK. ASK1 is a new member of the MAPKKK group and activates two different subgroups of MAPKK, SEK1 and MKK6, which in turn activate SAPK and p38. Overexpression of ASK1 reportedly induces apoptotic cell death (Ichijo *et al.*, 1997). Our findings identify a novel physiological role for cytoplasmic p21^{Cip1/WAF1} in inhibition of activation of the MAP kinase cascade and transformation to apoptosis-resistant cells.

Fig. 1. Normal human monocytes display cytoplasmic p21^{Cip1/WAF1} expression. **(A)** Dual staining of p21^{Cip1/WAF1} and CD14 in normal human monocytes. Cytospin preparations of blood mononuclear cells were stained for CD14 (green) and p21^{Cip1/WAF1} (p21C-Ab, red). Virtually all CD14-positive cells are also positive for the cytoplasmic p21^{Cip1/WAF1}. Bar, 10 μ m. **(B)** Western blot analysis of p21^{Cip1/WAF1} in nuclear and cytoplasmic fractions of CD14 positive monocytes. Cytoplasmic and nuclear extracts of CD14 positive monocytes were isolated and subjected to Western blot analysis using anti-p21^{Cip1/WAF1}, anti-p53 and anti-Bcl-2 antibodies.

Results

Normal human monocytes display cytoplasmic p21^{Cip1/WAF1} expression

p21^{Cip1/WAF1} functions as a cell cycle inhibitor, and this activity is closely associated with its nuclear localization in various tissues such as fibroblasts and epithelial cells (El-Deiry *et al.*, 1994; Halevy *et al.*, 1995; Andres and Walsh, 1996; LaBaer *et al.*, 1997). In the light of these previous findings, it was surprising to find p21^{Cip1/WAF1} localized in the cytoplasm of peripheral blood monocytes (PBMs) as demonstrated by the dual color immunofluorescence staining for the monocyte marker CD14 (Lubbert *et al.*, 1991) (FITC in Figure 1A) and for p21^{Cip1/WAF1} (Rhodamine in Figure 1A) using an antibody (#sc-397; p21C-Ab) for the C-terminal 19 amino acids. The specificity of these immunofluorescence data was verified by use of irrelevant control primary antibodies as well as by immunostaining with secondary antibody alone (data not

shown). The specificity of p21C-Ab for p21^{Cip1/WAF1} was verified in the present study (see below).

Expression of cytoplasmic p21^{Cip1/WAF1} in monocytes was demonstrated further by Western blot analysis of nuclear and cytoplasmic extracts prepared from CD14-positive blood mononuclear cells (MNCs) of healthy individuals (Figure 1B). Successful fractionation was verified by Western blot analysis for nuclear and cytoplasmic proteins using anti-p53 and anti-Bcl-2 antibodies, respectively (Figure 1B).

In vitro monocytic differentiation of U937 cells is associated with p21^{Cip1/WAF1} expression in cytoplasm

To investigate the biological role of cytoplasmic p21^{Cip1/WAF1} in monocytes, we used an *in vitro* monocyte differentiation system. It has previously been demonstrated that U937 cells differentiate into monocytes by treatment with vitamin D3 (VD3) (Bhalla *et al.*, 1989), a process mainly mediated by p21^{Cip1/WAF1} expression and subsequent G₀/G₁ cell cycle arrest (Liu *et al.*, 1996). p21^{Cip1/WAF1} induced by treatment of U937 with 10 nM VD3 for 1 day was located in the nucleus (Figure 2B). However, after 3 days of treatment, a time point when the monocytic differentiation was well evident, p21^{Cip1/WAF1} was mainly localized in the cytoplasm (Figure 2C), as determined by the expression of CD14 (Figure 3B). In this regard, the differentiation-associated cytoplasmic expression of nuclear p21^{Cip1/WAF1} was not restricted to U937 cells, since HL60 cells also demonstrated a similar distribution of p21^{Cip1/WAF1} upon differentiation induction with 10 nM VD3 for 3 days (Figure 2E and F). The presence of p21^{Cip1/WAF1} was confirmed by p21C-Ab (Figure 2).

Nuclear p21^{Cip1/WAF1} induces monocytic differentiation of U937 cells associated with cytoplasmic expression of itself during differentiation

VD3-induced differentiation of U937 cells is associated with the expression of several CDK inhibitors other than p21^{Cip1/WAF1} (Liu *et al.*, 1996). VD3 also has pleiotropic activities (Reichel *et al.*, 1989), which can be avoided in the *in vitro* differentiation system of U937 cells by ectopically expressing p21^{Cip1/WAF1}. In a stable U937 transfectant containing p21^{Cip1/WAF1} cDNA (U937/CB6-p21) as part of the heavy metal inducible vector pMT-CB6+ (Canman *et al.*, 1995), the addition of zinc (120 μ M of ZnSO₄) to the culture medium for between 6 h and 3 days induced p21^{Cip1/WAF1} expression, as determined by Western blot analysis using an anti-p21^{Cip1/WAF1} monoclonal antibody (#C24420; p21mAb) (Figure 3A). The specificity of this reaction band was further confirmed by two other anti-p21^{Cip1/WAF1} antibodies, #OP64C and p21C-Ab (data not shown). In contrast, U937/CB6-p21 cells cultured without zinc or U937-mock cells cultured without or with zinc did not express p21^{Cip1/WAF1} (Figure 3A). Fluorescence-activated cell sorting (FACS) analyses showed that U937/CB6-p21 cells, but not mock transfectants with vector alone, expressed CD14 (Bhalla *et al.*, 1989; Lubbert *et al.*, 1991) 3 days after the addition of zinc (Figure 3B). The level of CD14 expression was almost equivalent to that in the parental

Fig. 2. Subcellular localization of p21^{Cip1/WAF1} protein during VD3-induced monocytic differentiation. (A–F) Immunohistochemical staining of p21^{Cip1/WAF1} with p21C-Ab in U937 and HL-60 cells. Representative features of U937 cells cultured without VD3 (A), or with 10 nM VD3 for 1 day (B) or 3 days (C), and HL-60 cells cultured without VD3 (D), or with 10 nM VD3 for 1 day (E) or 3 days (F) are shown. (A) and (D) are phase-contrast images. Bars, 10 μ m.

U937 cells treated with 10 nM VD3 for 3 days (Figure 3B). Monocytic differentiation was also characterized by reactivity to anti-CD11b antibody and a positive reaction for the nitroblue tetrazolium (NBT) test (data not shown). These results are in accordance with those reported previously (Liu *et al.*, 1996) and indicate that p21^{Cip1/WAF1} expression plays by itself an important role in monocytic differentiation of U937 cells.

In the next step, we investigated the subcellular localization of p21^{Cip1/WAF1} using this *in vitro* differentiation system, which is directly regulated by p21^{Cip1/WAF1}. The reaction specificity of p21mAb for immunohistological analysis was verified in a panel of U937 cells with or without exogenously expressed p21^{Cip1/WAF1}. U937-mock cells showed no reactivity (Figure 3C), while U937/CB6-p21 cells showed reactivity only upon zinc treatment. After 6 h of zinc treatment in U937/CB6-p21 cells, a time point when no morphological differentiation was evident, p21^{Cip1/WAF1} was nuclear (Figure 3D), but after 3 days of zinc treatment, p21^{Cip1/WAF1} was expressed not only in the nucleus but also in the cytoplasm (Figure 3E). It was noted that while in some cells p21^{Cip1/WAF1} was expressed more in the nucleus than in the cytoplasm (Figure 3E, open arrow), in others it was the reverse (Figure 3E, closed arrow). The cytoplasmic expression of p21^{Cip1/WAF1} was also confirmed by p21C-Ab (Figure 3F).

These results imply that nuclear p21^{Cip1/WAF1} triggers monocytic differentiation. Furthermore, this process is associated with cytoplasmic translocation or retention of nuclear p21^{Cip1/WAF1}. It is also noted that cytoplasmic p21^{Cip1/WAF1} observed in our study is distinct from the recently described p21 Δ (Poon and Hunter, 1998). p21 Δ is a form of p21^{Cip1/WAF1} with deletion of $\sim$ 10 amino acids at the C-terminal and cannot be detected by p21C-Ab (Poon and Hunter, 1998). In contrast, our cytoplasmic p21^{Cip1/WAF1} is reactive with p21C-Ab (Figures 1, 2 and 3F), probably corresponding to the wild-type p21^{Cip1/WAF1}. As a control, HT1080 human fibrosarcoma cell line was stably transfected with pMT-CB6-p21 (HT/CB6-p21). HT/CB6-p21 cells showed persistent nuclear localization of p21^{Cip1/WAF1} by treatment with zinc for between 6 h (data not shown) and 3 days (Figure 3G) as determined by antibody p21mAb.

Differentiated U937 cells with cytoplasmic p21^{Cip1/WAF1} are resistant to apoptosis induced by apoptogenic stimuli

The correlation between p21^{Cip1/WAF1} status and sensitivity to apoptosis was examined in this system. An aliquot of cells with cytoplasmic p21^{Cip1/WAF1} (Figures 2 and 3) was analyzed for survival to various apoptogenic stimuli. Differentiated U937/CB6-p21 cells expressing

Fig. 3. Ectopic p21^{Cip1/WAF1} induces differentiation of U937 cells. **(A)** Western blot analysis of zinc-induced p21^{Cip1/WAF1} in U937/CB6-p21 cells. Whole-cell lysates prepared from cells cultured in the presence of zinc for the indicated time intervals (6h, 6 hours; 3d, 3 days) were analyzed by Western blot using anti-p21^{Cip1/WAF1} antibody (p21mAb). Note the lack of p21^{Cip1/WAF1} expression on U937-mock cells with or without zinc and U937/CB6-p21 cells without zinc treatment, while zinc treatment of the latter resulted in p21^{Cip1/WAF1} expression, indicating the specific activity of this antibody. **(B)** Enforced p21^{Cip1/WAF1} expression induces monocytic differentiation of U937/CB6-p21. FACS profiles of CD14 expression. U937-mock (top) and U937/CB6-p21 (middle) cells cultured for 3 days with (solid line) or without zinc (dashed line), and U937 cells cultured for 3 days in the presence (solid line) or absence (dashed line) of VD3 (bottom) are shown. Staining with control mouse IgG and FITC-conjugated anti-mouse IgG overlapped with broken lines. **(C–G)** Immunohistochemical staining of p21^{Cip1/WAF1} in U937-mock cells cultured with zinc **(C)**, U937/CB6-p21 cells cultured with zinc for 6 h **(D)** and 3 days **(E)**, and HT/CB6-p21 cells cultured with zinc for 3 days **(G)** are shown. In **(E)**, open and closed arrows indicate cells in which the expression of p21^{Cip1/WAF1} in the nucleus is higher and lower than in the cytoplasm, respectively. It should be noted that in U937/CB6-p21 treated with zinc for 3 days, cytoplasmic p21 was visualized by antibody p21C-Ab **(F)**. Inset in each panel is the phase-contrast feature of negative control with secondary antibody alone. Bars, 10 μ m.

cytoplasmic p21^{Cip1/WAF1} by 3-day treatment with zinc were extremely resistant to various inducers of apoptosis, such as hydrogen peroxide (H₂O₂), C2-ceramide and tumor necrosis factor (TNF) α . In contrast, U937-mock cells grown under similar experimental conditions were susceptible to apoptosis, as determined by subdiploid DNA contents after staining with propidium iodide (PI; Figure 4A). The sensitivity to apoptosis was also verified using a variety of other indicators of apoptotic cell death. DEVD-

sensitive caspase activation, determined using specific fluorogenic tetrapeptides, was detected 3 h after the addition of H₂O₂ (Figure 4B) and increased enzymatic activity was consistent with an elevation in fraction of apoptotic cells in 72 h zinc-treated U937-mock cells. Differentiated U937/CB6-p21 cells bearing cytoplasmic p21^{Cip1/WAF1} expression by 3-day zinc treatment, in contrast, showed no DEVD-sensitive caspase activation (Figure 4B), thus confirming the resistance to apoptosis

Fig. 4. Differentiated U937 cells are apoptosis resistant in association with cytoplasmic p21^{Cip1/WAF1} expression. **(A)** Percentage of apoptotic cells treated with TNF α (TNF), C2-ceramide (C2) or H₂O₂, in the clones with cytoplasmic p21^{Cip1/WAF1} protein. U937 cells were incubated with (VD3+) or without (VD3-) 10 nM VD3 for 3 days. U937/CB6-p21 (p21) and U937-mock (mock) cells were incubated with (Zn+) or without (Zn-) zinc for 3 days. Cells were cultured with 200 ng/ml human recombinant TNF α , 200 μ M C2-ceramide or 300 μ M H₂O₂ for 16 h. The apoptotic population identified as a subdiploid peak was analyzed by FACS. **(B)** Kinetics of caspase activation in zinc-differentiated U937/CB6-p21 cells stimulated by H₂O₂. YVAD-sensitive and DEVD-sensitive caspase activity in cell lysates of U937-mock (mock) and U937/CB6-p21 (p21) cultured in the presence of zinc for 3 days was determined. **(C)** SAPK/JNK activities in response to stimulation with H₂O₂ in the corresponding clones determined for subcellular localization of p21^{Cip1/WAF1} protein. U937/CB6-p21 cells were cultured in the absence or presence of zinc for between 6 h and 3 days and then mock-treated or treated with 300 μ M H₂O₂ for 2 h. Aliquots of cells were cultured for 2 h in the presence of 300 μ M H₂O₂. They were subjected to *in vitro* kinase assay using human recombinant ATF-2 as a substrate (top). Fold, fold-activation standardizing background SAPK/JNK activity as 1. The level of SAPK/JNK immunoprecipitated by anti-SAPK/JNK antibody was determined by Western blotting (bottom).

in these cells. No YVAD-sensitive caspase activity was detected in any of the transfectants used in this study. Apoptosis-resistant phenotype of differentiated U937 cells was supported further by loss of reduction of mitochondrial transmembrane potential ($\Delta\Psi$ m) using a mitochondrial

transmembrane potential sensitive dye DiOC₆ (3) (Zoratti and Szabo, 1995) (data not shown). U937 cells induced to differentiate for 72 h with VD3 also showed an apoptosis-resistant phenotype (Figure 4A).

SAPK/JNK is a stress-activated MAP kinase involved in transmitting proapoptotic signals (Verheij *et al.*, 1996). We therefore studied the activation of this kinase to determine its relationship with the differentiation status. Additionally, given that p21^{Cip1/WAF1} inhibits the catalytic activity of SAPK/JNK (Shim *et al.*, 1996) and that activation of the latter is associated with a shift from the cytoplasm to the nucleus (Cavigelli *et al.*, 1995), it was important to study the correlation between subcellular localization of p21^{Cip1/WAF1} and SAPK/JNK activation. Zinc-treated U937-mock cells, negative for p21^{Cip1/WAF1} and U937/CB6-p21 cells expressing nuclear p21^{Cip1/WAF1} after 6 h zinc induction, both demonstrated a significant activation of SAPK/JNK (Figure 4C). This was also the case in HT/CB6-p21 cells with zinc-induced expression of nuclear p21^{Cip1/WAF1} (after culture for 3 days) (Figure 4C). In contrast, differentiated U937/CB6-p21 cells expressing cytoplasmic p21^{Cip1/WAF1} by 3-day zinc treatment showed no SAPK/JNK activation (Figure 4C). These results suggest that the subcellular localization of p21^{Cip1/WAF1} is relevant to SAPK/JNK activation and cellular sensitivity to apoptosis.

Cytoplasmic p21^{Cip1/WAF1} with loss of nuclear localization signal (Δ NLS-p21) is a potent inhibitor of apoptosis

Since the mechanism of cytoplasmic expression of p21^{Cip1/WAF1} during monocytic differentiation of U937 cells is not known and p21^{Cip1/WAF1} is a nuclear protein with bipartite nuclear localization signal (NLS), we cannot construct an experimental model with cytoplasmic expression of the wild-type p21^{Cip1/WAF1} bearing NLS. Instead, we subcloned a mutant cDNA that lacked the nuclear localization signal of p21^{Cip1/WAF1} (Δ NLS-p21; aa 1–140) in pMT-CB6 vector (CB6- Δ NLS-p21). U937/CB6- Δ NLS-p21, a stable transfectant of U937 with CB6- Δ NLS-p21, was treated with zinc. Expression of Δ NLS-p21 was confirmed by a Western blot analysis showing a faster migration compared with full-length (aa 1–164) p21^{Cip1/WAF1} in U937/CB6-p21 (Figure 5A). *In situ* immunohistochemical analysis demonstrated that Δ NLS-p21 expression was mainly cytoplasmic (Figure 5B). It should be noted that a C-terminus specific anti-p21^{Cip1/WAF1} antibody (p21C-Ab) failed to detect Δ NLS-p21 (Figure 5C) but not cytoplasmic p21^{Cip1/WAF1} in U937/CB6-p21 (Figure 3F). These results indirectly verified the specificity of p21C-Ab demonstrated in Figure 1. Cytoplasmic Δ NLS-p21-expressing cells showed either no differentiation or cell cycle arrest after 72 h of zinc treatment (Figure 5D and E, respectively), and this could not be ascribed to a specific defect in this particular U937/CB6- Δ NLS-p21 clone since VD3 treatment induced monocytic differentiation and G₀/G₁ arrest in this clone (Figure 5D and E, bottom panels).

We examined the response of U937/CB6- Δ NLS-p21 cells incubated for 72 h with zinc and thereafter exposed to various apoptogenic agents, including C2-ceramide, H₂O₂, TNF α or X-ray irradiation, and found that these

Fig. 5. U937/CB6-ΔNLS-p21 cells did not show cell cycle arrest or cell differentiation. (A) Western blot analysis of p21^{Cip1/WAF1} in U937/CB6-ΔNLS-p21 cells. Whole-cell lysates from U937/CB6-p21 cells (lane 1), U937/CB6-ΔNLS-p21 cells (lane 2) and U937-mock cells (lane 3) cultured in the presence of zinc for 3 days were subjected to Western blot analysis for p21^{Cip1/WAF1} protein. Due to the deletion of 24 aa, ΔNLS-p21 protein runs faster than full-length p21^{Cip1/WAF1}. (B and C) Immunohistochemical staining of p21^{Cip1/WAF1} in U937/CB6-ΔNLS-p21 cells. U937/CB6-ΔNLS-p21 cells cultured with zinc for 3 days were stained with p21mAb (B) or p21C-Ab (C) antibody. Inset is a negative control. Bar, 10 μm. (D and E) U937/CB6-ΔNLS-p21 cells exhibit neither cell differentiation nor cell cycle arrest. U937/CB6-ΔNLS-p21 cells were cultured with (middle) or without (top) zinc, or in the presence of VD3 (bottom) for 3 days. FACS profiles of CD14 expression are shown in (D). Cell cycle distribution of U937/CB6-ΔNLS-p21 and U937/CB6-p21 cells is shown in (E). Cells were analyzed for DNA content by PI staining. The percentages of cells at G₀/G₁, S and G₂/M are indicated.

cells are extremely resistant, as determined by the cell fractions with subdiploid DNA contents (Figure 6A). Such resistance was also validated by several other indicators. Loss of ΔΨ_m in response to C2-ceramide stimulation was suppressed in U937 cells expressing ΔNLS-p21 (Figure 6B). Likewise, SAPK/JNK (Figure 6C) and DEVD-sensitive caspase activation (data not shown) in response to H₂O₂ were also inhibited in these cells. Furthermore, HT1080 cells expressing cytoplasmic ΔNLS-p21 (HT/CB6-ΔNLS-p21), but not HT-mock or HT/CB6-p21 with nuclear expression of p21^{Cip1/WAF1}, were also resistant to 1 μM staurosporine, another potent inducer of apoptosis (Figure 6D). These results confirmed our suspicion that cytoplasmic p21^{Cip1/WAF1} is directly responsible for inhibition of

apoptosis. Furthermore, this is not a phenomenon restricted to U937 cells.

Cytoplasmic p21^{Cip1/WAF1} forms a physical complex with ASK1 and inhibits upstream of MAP kinase activation

The reduction of ΔΨ_m is an event that occurs before the activation of DEVD-sensitive caspase in the apoptotic signal cascade (Susin *et al.*, 1997). Whether the activation of SAPK/JNK precedes or follows the reduction of ΔΨ_m is unknown. To clarify this issue and to define further the sequence of events in the pro-apoptotic signal cascade, we have examined the kinetics of SAPK/JNK activation and the reduction of ΔΨ_m in our system. Activation of SAPK/JNK was detected 1 h after the addition of

Fig. 6. U937/CB6-ΔNLS-p21 cells are resistant to apoptogenic agents. (A) U937/CB6-ΔNLS-p21 cells cultured in the presence of zinc show resistance to the apoptosis induced by C2-ceramide, TNF α , H₂O₂ or X-ray irradiation (IR). U937-mock and U937/CB6-ΔNLS-p21 cells were treated with 200 ng/ml TNF α (TNF), 200 μ M C2-ceramide (C2), 300 μ M H₂O₂ or 20 Gy of X-ray IR, and were stained by PI. (B) U937/CB6-ΔNLS-p21 cells cultured in the presence of zinc are resistant to the reduction of $\Delta\Psi$ m induced by C2-ceramide (C2). U937-mock and U937/CB6-ΔNLS-p21 cells were incubated with (Zn+) or without (Zn-) zinc for 3 days, resuspended in a medium containing 200 μ M C2-ceramide. Reductions in $\Delta\Psi$ m were analyzed by FACS. (C) SAPK/JNK activities in response to H₂O₂ stimulation in U937/CB6-ΔNLS-p21 cells. Cells were cultured in the absence or presence of zinc for 3 days and then mock treated or treated with 300 μ M H₂O₂ for 2 h. They were subjected to *in vitro* kinase assay as in Figure 4C. Fold, fold-activation standardizing background SAPK/JNK activity as 1. (D) HT/CB6-ΔNLS-p21 cells cultured in the presence of zinc are resistant to the reduction of $\Delta\Psi$ m induced by 1 μ M staurosporine. HT-mock, HT/CB6-p21 and HT/CB6-ΔNLS-p21 cells cultured with zinc for 3 days were incubated in the absence (broken line) or presence (solid line) of 1 μ M staurosporine for 16 h and were analyzed for their reduction of $\Delta\Psi$ m by FACS.

H₂O₂, while a reduction in $\Delta\Psi$ m was delayed by 1–2 h (Figure 7A and B). Bongkreikic acid (BA), a specific inhibitor of mitochondrial permeability transition (Marchetti *et al.*, 1996), inhibited the reduction in $\Delta\Psi$ m (Figure 7D) and subsequent apoptosis induced by H₂O₂ but failed to block SAPK/JNK activation (Figure 7C). These results suggest that cytoplasmic p21^{Cip1/WAF1} either inhibits the activation of SAPK/JNK as well as reduction of $\Delta\Psi$ m independently, or prevents a reduction of $\Delta\Psi$ m through the inhibition of SAPK/JNK activation.

Shim *et al.* (1996) have demonstrated recently that p21^{Cip1/WAF1} inhibits SAPK/JNK activation and speculated that p21^{Cip1/WAF1} may inhibit upstream of SAPK/JNK activation. This finding is in agreement with our results of inhibition of SAPK/JNK activation in cells with cytoplasmic expression of p21^{Cip1/WAF1}. These findings prompted us to investigate the interaction between p21^{Cip1/WAF1} and ASK1, a member of the MAPKKK group which activates two different subgroups of MAPKK lying upstream of SAPK activation. Using specific antiserum to ASK1 (Ichijo *et al.*, 1997), p21^{Cip1/WAF1} was detected in the immune complexes of ASK1 from differentiated U937/CB6-p21 cells expressing cytoplasmic p21^{Cip1/WAF1} (Figure 8A, lane 2) and U937 cells with cytoplasmic ΔNLS-p21 (Figure 8A, lane 3), but not from U937-mock cells (Figure

8A, lane 1). The finding that cytoplasmic p21^{Cip1/WAF1} interacts with ASK1 is compatible with the cytoplasmic localization of ASK1 when overexpressed (H.Ichijo and T.Hamazaki, unpublished data). The issue of where the subcellular fractions ASK1 and p21^{Cip1/WAF1} interact was investigated further using 293 cells transfected with green fluorescent protein (GFP)-fused p21^{Cip1/WAF1} with or without NLS (GFP-p21-full or GFP-ΔNLS-p21, respectively). 293 cells transfected with GFP-p21-full demonstrated exclusive nuclear localization of GFP, and cytoplasmic expression of GFP was well evident in cells transfected with GFP-ΔNLS-p21 (data not shown). When immunoprecipitated with anti-GFP antibody, ASK1 was demonstrated in cells transfected with GFP-ΔNLS-p21 but not in cells with GFP-p21-full (Figure 8B). These results support the notion that they interact in the cytoplasm but not in the nucleus. Furthermore, activation of ASK1 kinase, as determined by using MKK6 for a substrate, which was evident 15 min following the addition of H₂O₂ in U937-mock cells, was blocked in U937 cells expressing ΔNLS-p21 (Figure 8C). These findings together with the inhibition of activation of SAPK/JNK suggest that one of the targets of action of cytoplasmic p21^{Cip1/WAF1} and ΔNLS-p21 involves ASK1, which in turn interferes with the apoptosis-signaling MAP kinase pathway.

Fig. 7. SAPK/JNK activation is independent of reduction in mitochondrial $\Delta\Psi_m$. (A) Kinetics of SAPK/JNK activation after treatment with H₂O₂. Lysates isolated from U937 cells cultured in the presence of H₂O₂ were subjected to *in vitro* kinase assay. Fold, fold-activation standardizing background SAPK/JNK activity as 1. (B) Kinetics of $\Delta\Psi_m$ reduction after treatment with H₂O₂. U937 cells cultured in the presence of H₂O₂ for 0, 1, 2, 3, 4, 6 and 24 h were analyzed by FACS. (C) Treatment with BA does not inhibit SAPK/JNK activation. U937 cells cultured in the presence of 200 μ M BA, 50 μ M H₂O₂ or both for 1.5 h were subjected to *in vitro* kinase assay. Fold, fold-activation standardizing background SAPK/JNK activity as 1. (D) BA inhibits H₂O₂-induced reduction in $\Delta\Psi_m$. U937 cells cultured in the presence of 200 μ M BA, 50 μ M H₂O₂ or both for 16 h were analyzed by FACS. In spite of SAPK/JNK activation at 1.5 h after H₂O₂ stimulation (C), U937 cells show significant inhibition of $\Delta\Psi_m$ reduction in the presence of BA.

Cytoplasmic p21^{Cip1/WAF1} interacts with ASK1 in normal human monocytes

Physical interaction between cytoplasmic p21^{Cip1/WAF1} and ASK1 in monocytes was investigated by the pull-down assays. Cytoplasmic p21^{Cip1/WAF1} expressed in monocytes was co-immunoprecipitated with antiserum to ASK1 and visualized by p21mAb (Figure 8A, lane 4) as well as p21C-Ab (data not shown). These results suggest that p21^{Cip1/WAF1} in monocytes may inhibit proapoptotic signals mediated by stress-activated MAP kinase cascade.

Discussion

The major finding of our study was that mature monocytes express the cell cycle inhibitor p21^{Cip1/WAF1} in the cytoplasm. Our *in vitro* system for the differentiation of U937 cells either by VD3 treatment or by ectopic expression of p21^{Cip1/WAF1} also indicated that monocytic differentiation is associated with a cytoplasmic translocation or retention of nuclear p21^{Cip1/WAF1}. Our findings were rather unexpected, since p21^{Cip1/WAF1} has consistently been shown to be localized in the nucleus (El-Deiry *et al.*, 1994) and to have a functional role in the inhibition of cyclin/CDK and PCNA activities. Together, our results based on U937 as a model system of monocytic differentiation and on fresh PBMs suggest that nuclear expression of p21^{Cip1/WAF1} and subsequent G₁ cell cycle arrest may allow the differentiation program already set in monocytic precursor cells to proceed. While this differentiation program takes place, the nuclear localization of p21^{Cip1/WAF1} becomes cytoplasmic.

An apoptosis-resistant phenotype appeared concomitantly with the expression of cytoplasmic p21^{Cip1/WAF1}. Recent studies investigating the apoptotic process have

indicated the presence of three sequential stages of apoptosis such as initiation, effector and degradation (Kroemer, 1997). To elucidate how differentiation-associated expression of cytoplasmic p21^{Cip1/WAF1} inhibits apoptosis, we sequentially investigated several markers of apoptosis, including caspase activation, reduction of $\Delta\Psi_m$ and activation of SAPK/JNK. In H₂O₂-induced apoptosis, activation of SAPK/JNK precedes $\Delta\Psi_m$ reduction (Figure 7A and B), whereas the latter appears to occur before the activation of DEVD-sensitive caspase (Susin *et al.*, 1997), both of which are thought to occur during the effector phase (Kroemer *et al.*, 1997). Since SAPK/JNK is activated without loss of $\Delta\Psi_m$ in the presence of BA (Figure 7C and D), these findings support the hypothesis that activation of the MAP kinase cascade precedes reduction of $\Delta\Psi_m$. These results are in agreement with our finding that ASK1, which is a member of the MAPKKK group and activates two different subgroups of MAPKK, SEK1 and MKK6, and SAPK/JNK are already activated by the time the loss of $\Delta\Psi_m$ is detected 3–4 h after H₂O₂ stimulation (Figures 8C and 7A, respectively). Since p21^{Cip1/WAF1} associates physically with ASK1 and inhibits the activation of SAPK/JNK, p21^{Cip1/WAF1}-mediated inhibition of apoptosis may be targeted at the initial phase of this process. Recently MEKK-1, a member of the MAPKKK group, has been shown to be activated by caspase 7, implying the involvement of a positive feedback loop between these molecules (Cardone *et al.*, 1997). Thus, our findings suggest that the complex formation of cytoplasmic p21^{Cip1/WAF1} with ASK1 is directly responsible for resistance to apoptosis by inhibition of activation of the MAP kinase cascade, which lies in the initial phase of signal transduction and possibly

Fig. 8. p21^{Cip1/WAF1} interacts with ASK1 and blocks its activity. **(A)** Cytoplasmic p21^{Cip1/WAF1} was co-immunoprecipitated by antiserum to ASK1. U937-mock (lane 1), U937/CB6-p21 (lane 2), and U937/CB6-ΔNLS-p21 (lane 3) cells were cultured in the presence of zinc for 3 days. Cell lysates from each sample and peripheral blood CD14 positive monocytes (lane 4) were immunoprecipitated by antiserum to ASK1, then subjected to Western blot analysis with p21mAb. Whole-cell lysate from p21^{Cip1/WAF1} expressing HT/CB6-p21 (lane 5) was a size marker for p21^{Cip1/WAF1}. **(B)** ASK1 interacts with cytoplasmic p21, but not with nuclear p21 in 293 cells. 293 cells were transiently transfected with GFP-fused p21, either without NLS (GFP-ΔNLS-p21) (lanes 1 and 3) or with NLS (GFP-p21-full) (lanes 2 and 4). Expression of GFP-fused p21 was demonstrated by αGFP Ab (lanes 1 and 2) and p21mAb (lanes 3 and 4). GFP immunocomplex was analyzed by Western blot using αASK1 antibody (right). p21 was expressed exclusively in the nucleus in 293 cells transfected with GFP-p21-full, while cytoplasmic expression of p21^{Cip1/WAF1} was evident in GFP-ΔNLS-p21 transfectant as examined under the fluorescence microscope (data not shown). GFP immunocomplex from 293 cells transfected with GFP vector alone did not display ASK1 interaction (data not shown). **(C)** Inhibition of H₂O₂-induced ASK1 activation in cytoplasmic p21^{Cip1/WAF1} expressing cells. Top; U937-mock or U937/CB6-ΔNLS-p21 cells were cultured in the presence of zinc for 3 days and then mock-treated or treated with 300 μM H₂O₂ for 15 min, and then subjected to *in vitro* kinase assay. Bottom, relative ASK1 kinase activity in response to H₂O₂ treatment in cells with or without cytoplasmic p21^{Cip1/WAF1} expression. Changes in ASK1 kinase activity of cells treated with H₂O₂ were calculated by standardizing the activity without H₂O₂ treatment to 1. Results are the mean ± sd of three separate experiments.

in the positive amplification loop in a cascade of apoptotic processes.

More direct evidence that the apoptosis-resistant phenotype was raised by cytoplasmic p21^{Cip1/WAF1} rather than by some unknown mechanisms associated with cell cycle arrest or cell differentiation was provided in our study using the ΔNLS-p21 mutant. Expression of cytoplasmic ΔNLS-p21 rendered the cells apoptosis resistant, participated in a complex formation with ASK1 (Figure 8A), and inhibited the activation of ASK1 (Figure 8C) and SAPK/JNK (Figure 6C). Since ΔNLS-p21 does not induce cell differentiation or cell cycle arrest, neither of these processes is likely to be involved in the induction of apoptosis-resistant phenotype. Recent studies have shown that thioredoxin (Trx) is a direct inhibitor of ASK1 (Saitoh *et al.*, 1998); however, the mechanism of inhibition of ASK1 by this compound seems different from that of cytoplasmic p21^{Cip1/WAF1}. Trx can block ASK1 activation only under reducing conditions; in contrast, even in the presence of H₂O₂, cytoplasmic p21^{Cip1/WAF1} could inhibit ASK1 activation. Further studies are currently underway to examine how cytoplasmic p21 inhibits activation of ASK1.

During the preparation of this manuscript, Poon and Hunter (1998) described a novel form of p21 (p21Δ) in the cytoplasm of UV-irradiated normal diploid fibroblasts and tumor cells. p21Δ is characterized by loss of

~10 amino acids at the C-terminal end and localizes in the cytoplasm due to insufficient signals for nuclear localization. Based on this new finding, we investigated whether the differentiation-associated cytoplasmic p21^{Cip1/WAF1} corresponds to p21Δ. While the antibody specific for the C-terminus of human p21^{Cip1/WAF1} (p21C-Ab) is unreactive with ΔNLS-p21 (Figure 5C) or p21Δ (Poon and Hunter, 1998), it was reactive with cytoplasmic p21^{Cip1/WAF1} in differentiated U937 and PBMs. Thus, the cytoplasmic p21^{Cip1/WAF1} demonstrated in our study is very unlikely to correspond to p21Δ. It is not clear at present whether the differentiation-associated cytoplasmic p21^{Cip1/WAF1} preserves an intact form of NLS or contains insufficient construct of NLS which cannot be recognized by our Western blot analysis or p21C-Ab. In the light of no apparent loss of C-terminal sequences, however, the differentiation-associated cytoplasmic p21^{Cip1/WAF1} may employ a mechanism distinct from deletions or mutations of NLS. In this respect, we speculate that modulation of the nuclear import or export signal for p21^{Cip1/WAF1} is involved in the process of monocytic differentiation.

Expression of p21^{Cip1/WAF1} in the cytoplasm of PBMs was demonstrated in the present study by immunohistochemical analysis as well as by Western blotting on nuclear and cytoplasmic compartments. Since cytoplasmic

p21^{Cip1/WAF1} was co-immunoprecipitated with ASK1 in monocytes, this must have an important physiological role in protecting monocytes against apoptogenic stimulation. Monocytes destroy intracellular pathogens and extracellular targets in part by the production of toxic oxygen metabolites, including superoxide, hydrogen peroxide, hydroxyl radicals and possibly singlet molecular oxygen (Klebanoff *et al.*, 1983). This type of toxicity requires the production of reactive oxygen species by phagocytes in quantities sufficient to overcome the protective capacity of endogenous scavengers in the parasite. In the case of monocytes, the cytoplasmic p21^{Cip1/WAF1} expression may support survival of monocytes in the presence of oxidative stresses, thereby enabling these cells to accomplish their specified functions.

Taken together, our findings suggest that nuclear p21^{Cip1/WAF1} is physiologically expressed in the cytoplasm during the development of monocytes. Furthermore, p21^{Cip1/WAF1}, originally identified as a cell cycle inhibitor, acts as an inhibitor of apoptosis in the cytoplasm. Further studies are currently being conducted in our laboratories to elucidate molecular mechanisms involved in the differentiation-associated cytoplasmic translocation or retention of CDK inhibitor p21^{Cip1/WAF1}.

Materials and methods

Cell culture, antibodies and reagents

Cells were cultured in RPMI 1640 (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL) in 5% CO₂ environment at 37°C. Monoclonal anti-p21^{Cip1/WAF1} antibodies #C24420 (p21mAb) and #OP64C were purchased from Transduction Laboratories, KY, and Oncogene Research Products (Cambridge, MA), respectively. The polyclonal anti-p21^{Cip1/WAF1} antibody #sc-397 (p21C-Ab) and polyclonal anti-SAPK/JNK antibody (#sc-474) were purchased from Santa Cruz Biotechnology, Inc., CA. Monoclonal anti-p53 antibody (DO-7.7, #M7001) was purchased from DAKO, Inc. (Tokyo, Japan). Monoclonal anti-Bcl-2 antibody (#OP60) was purchased from Oncogene Research Products (Cambridge, MA). Monoclonal anti-CD14 antibody (CLB-Mon/1) was purchased from Nichirei Co., Tokyo. 1,25-dihydroxyvitamin D3 was kindly provided by Dulphar (Amsterdam, The Netherlands). C2-ceramide was purchased from Wako (Tokyo). TNF α was a kind gift from Dai-Nippon Pharmaceutical Co., Tokyo. Ac-DEVD-MCA, Ac-YVAD-MCA and AMC (a reference compound for analysis with peptidyl-MCAs) were purchased from Peptide Institute, Inc. (Osaka, Japan). DiOC₆ (3) was purchased from Molecular Probe Co. (Eugene, OR). BA was kindly provided by Dr Duine (Delft University of Technology, Delft, The Netherlands). Antiserum to ASK1 was generated against the peptide sequence TEEKGRSTEEGDCESD (aa 654–669 of ASK1) that was coupled to keyhole limpet hemocyanin, and its reaction specificity has been described previously (Ichijo *et al.*, 1997). Polyclonal anti-GFP antibody was purchased from Clontech Laboratory, Inc. (Palo Alto, CA).

Isolation of monocytes from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (<1.077 g/cm³; Ficoll-Paque: Pharmacia Fine Chemicals, Piscataway, NJ). PBMCs were then incubated with a monocyte-specific monoclonal antibody to CD14, washed twice, and monocytes were isolated by magnetic separation on MACS columns (Miltenyi Biotec, Germany) using the procedure recommended by the manufacturer. Purity of the recovered cells (>95%) was checked by morphology and by immunofluorescence staining with a fluorescein-labeled anti-mouse antibody.

Cytoplasmic and nuclear fractionation

The monocytes were pelleted and resuspended in 300 μ l of buffer A (50 mM NaCl, 10 mM HEPES pH 8, 500 mM sucrose, 1 mM EDTA, 0.5 mM Spermidine, 0.15 mM Spermine, 0.2% Triton X-100) containing β -mercaptoethanol and the protease inhibitors PMSF, leupeptin, aprotinin

and pepstatin. After 15 min on ice and centrifugation, the supernatant (cytoplasmic fraction) was collected and stored while the pellet was washed with 200 μ l Buffer B (50 mM NaCl, 10 mM HEPES pH 8, 25% glycerol, 0.1 mM EDTA, 0.5 mM Spermidine, 0.15 mM Spermine) and then resuspended in 100 μ l buffer C (350 mM NaCl, 10 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM Spermidine, 0.15 mM Spermine). After centrifugation, the supernatant (nuclear fraction) was recovered. Cytoplasmic and nuclear fractions were quantitated for protein content by micro-BCA method (Pierce) and subjected to Western blot analysis.

Western blot analyses

Cells were suspended in a three-detergent lysis buffer (150 mM NaCl, 1.0% NP-40, 0.1% SDS, 1.0% sodium deoxycholate, 5 mM EDTA, 10 mM Tris, pH 7.4) containing protease inhibitors, and quickly sonicated on ice. Protein concentrations were measured using a commercial DC Protein Assay (Bio-Rad). Thirty micrograms of cytoplasmic or nuclear protein/lane was electrophoresed on 12.5% SDS-PAGE, or 30 μ g of soluble total cellular protein was electrophoresed in a Multi Gel 15/25 (Daiichi Pure Chemicals Co., Tokyo), and were electroblotted thereafter on PVDF membranes (Amersham Japan, Tokyo). Binding of the primary antibody was detected using a commercial ECL kit (Amersham, Japan).

In situ immunohistochemistry of p21^{Cip1/WAF1}

Dual staining of p21^{Cip1/WAF1} and CD14 was as follows. Anti-p21^{Cip1/WAF1} rabbit antibody (p21C-Ab) and anti-CD14 mouse antibody were simultaneously loaded on the acetone-fixed cytospin material. After extensive washing, the samples were incubated with biotinylated goat anti-rabbit IgG, followed by incubation with streptavidin-Rhodamine. In the next step, a blocking reaction against free biotin and avidin was conducted by using an endogenous avidin/biotin blocking kit (Nichirei, Tokyo) according to the manufacturer's protocol. After extensive washing in phosphate-buffered saline (PBS), samples were incubated with biotinylated rabbit anti-mouse IgG, followed by incubation with avidin-fluorescein isothiocyanate (FITC). Non-specific reaction of avidin-FITC by binding to free biotin was ruled out by a negative fluorescence in a reaction with avidin-FITC without incubation with biotinylated anti-mouse IgG. Non-specific reaction of biotinylated anti-mouse IgG by binding to free avidin was ruled out by a negative fluorescence in a reaction with biotinylated anti-rat IgM followed by avidin-FITC. Samples were examined under the fluorescence microscope (Olympus BH2) or confocal laser scanning microscope (Olympus LSM-GB200).

The alkaline phosphatase or peroxidase-based detection method was employed for immunohistochemical staining of p21^{Cip1/WAF1} in U937 cells. This is due to the high fluorescence background of U937 cells. Cells were fixed with 60% buffered formol acetone and subjected to *in situ* immunohistochemistry. Endogenous peroxidase or alkaline phosphatase was inactivated by treatment with 0.3% H₂O₂ or 1 mM levamisole, respectively. Binding of the primary antibody was detected using a commercial ABC kit (Vector Laboratories, Inc., Burlingame, CA) or Histofine SAB-AP kit (Nichirei, Tokyo). Monoclonal antibody p21mAb or polyclonal antibody p21C-Ab was employed as anti-p21^{Cip1/WAF1} antibody.

Plasmid construction and transfection

p21^{Cip1/WAF1} cDNA (corresponding to aa 1–164) was obtained by PCR amplification using a TPA-treated U937 cDNA as template and a set of primers 5'-GGAAGCTTCCTGCCGAAGTCAGTTCCTTGTTGA-3' and 5'-CCAAGCTTCCTGTGGCGGATTAGGGCTT-3'. p21^{Cip1/WAF1} cDNA was cloned as a *Hind*III fragment into a zinc-inducible pMT-CB6+ eukaryotic expression vector which contains cDNA under the control of a sheep metallothioneine promoter, and neomycin resistance gene driven by the SV40 early promoter. For the Δ NLS-p21 (aa 1–140), we used primers 5'-GGAAGCTTCCTGCCGAAGTCAGTTCCTTGTTGA-3' and 5'-GGTCTAGATCGACCCTGAGAGTCTCCAGG-3'. The nucleotide sequence and orientation of the inserted DNA was confirmed by sequencing. The vector DNAs were electroporated into U937 cells and transfected using Lipofectamine (Life Technologies) into HT1080 cells. Neomycin-resistant clones were isolated in media containing G418 (2 mg/ml) for 3 weeks and then tested in the presence of 120 μ M of ZnSO₄ (to induce each p21^{Cip1/WAF1} protein) by immunoblot analysis using antibody against p21^{Cip1/WAF1}.

The plasmid vector for GFP-fused p21 expression was constructed in pEGFP-C3 (Clontech). For transient expression, either pEGFP-p21-full or pEGFP- Δ NLS-p21 was electroporated into 293 cells. After 24 h cells were collected for immunoblot analysis.

Flow cytometric analysis

Flow cytometric studies of cell-surface antigens were performed according to standard techniques, using monoclonal antibody directed against the monocytic antigen CD14. Antibody binding was detected with a fluorescent rabbit anti-mouse antibody (Wako, Tokyo). Flow cytometry was performed on a Becton-Dickinson FACSsort.

For DNA staining analysis, U937 cells were washed in ice-cold PBS (Mg^{2+} - and Ca^{2+} -free, 1% bovine serum albumin) and permeabilized with 70% ethanol at $-20^{\circ}C$ for 20 min. Cells were washed with PBS twice, and the cell pellets were resuspended in 1×10^6 /ml of $1 \times$ PBS, treated with RNase A (50 μ g/ml) at $37^{\circ}C$ for 30 min, and stained with PI (5 μ g/ml). Flow cytometry was performed on a Becton-Dickinson FACSsort. For analysis of apoptosis, we determined the number of U937 cells with subdiploid DNA contents. Data were expressed as percentage of apoptotic cells relative to the total number of cells. For cell cycle analysis, data were processed using the ModFit LT™ software (Verity Software House, Inc.). For analysis of mitochondrial membrane potential, 1×10^6 /ml cells were incubated in the presence of 40 nM DiOC₆ (3) for 15 min at $37^{\circ}C$. Cells were then washed with PBS and subjected to FACS analysis. Cells with reduced $\Delta\psi_m$ were defined as apoptotic cells.

Immunoprecipitation and in vitro kinase assays

Cells were suspended at 3×10^6 /ml in lysis buffer (20 mM Tris-HCl pH 7.5, 12 mM β -glycerophosphate, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mM DTT) containing protease inhibitors, and quickly sonicated on ice. Immune complexes were immunoprecipitated from clarified cell lysates with protein A/G-agarose (protein A/G plus agarose, #sc2003, Santa Cruz) precoated with antibody to SAPK/JNK or antiserum to ASK1. Agarose was washed twice with wash solution I (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EGTA, 1% Triton-X-100, 2 mM DTT, 1 mM PMSF), then twice with wash solution II (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EGTA, 2 mM DTT, 1 mM PMSF). For kinase assay, immune complex was incubated for 30 min at $30^{\circ}C$ with substrate, in 25 μ l of kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) supplemented with 50 μ M ATP, 10 μ Ci of [γ -³²P]ATP (Amersham). Substrates used included 0.5 μ g of ATF2 (ATF-2[1-505], #sc4007, Santa Cruz) for SAPK/JNK and GST-MKK6KN (bacterially expressed catalytically inactive MKK6) for ASK1. Data are representative of at least three independent experiments.

Analysis of caspase activity

The activities of DEVD-sensitive and YVAD-sensitive cysteine proteases were analyzed using Ac-DEVD-MCA or Ac-YVAD-MCA as a substrate, respectively. Cells were suspended in lysis buffer (0.5% NP-40, 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris, pH 7.5) and kept on ice for 30 min, then centrifuged and supernatants collected. Enzyme reactions were performed in a buffer (10 mM HEPES, 0.1 M NaCl, 5 mM DTT) supplemented with 100 μ M of Ac-DEVD-AMC or Ac-YVAD-AMC at $37^{\circ}C$ for 2 h. The released fluorescent AMC was measured by a fluorescence spectrophotometer F-2000 (Hitachi co., Japan) with excitation/emission wavelengths of 365/450 nm, respectively. One unit was defined as the amount of enzyme that liberated 10 μ mol of AMC over 2 h.

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