Rac1 prevents cisplatin-induced apoptosis through down-regulation of p38 activation in NIH3T3 cells

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Abstract In this study, the role of V12-Rac1 in the cisplatin-induced apoptosis was investigated. Cisplatin-induced apoptosis is associated with cytochrome *c* release, which can be inhibited by V12-Rac1 expression. The analysis of mitogen-activated protein kinase activity indicated that V12-Rac1 expression led to a decrease in p38 activity after exposure to cisplatin but not c-jun N-terminal kinase and extracellular signal-regulated kinase. Using pharmacological inhibitors, it was found that only p38 is a critical mediator in the cisplatin-induced apoptosis of NIH3T3 cells. This suggests that V12-Rac1 can stimulate the anti-apoptotic signaling pathway in response to cisplatin, and that decreased p38 activity caused by V12-Rac1 expression in cisplatin-treated NIH3T3 cells is crucial for V12-Rac1-dependent cell survival. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Rac1; Rho family; Cisplatin; Apoptosis; p38; Cytochrome *c*

1. Introduction

Rac is activated by growth factors and transmits these signals to the downstream effectors [1–3]. Rac activation induces a reorganization of the actin cytoskeleton, cell cycle progression, gene transcription, and membrane transport [4]. Rac has also been shown to modulate the apoptosis induction pathway, resulting in either pro-survival or pro-apoptotic functions. Although a number of studies concerning the effects of Rac on modulating apoptosis have been reported, the role of Rac in modulating apoptosis is unclear. Several studies reported that Rac stimulates Bcl-2 expression in T and epithelial cells [5,6], and constitutive Rho GTPases activation prevents UV-induced apoptosis in epithelial cells [7]. Similarly, constitutively active Rac provides a survival signal under various stimuli in neurons, fibroblasts, epithelial cells and hematopoietic cells [8–13]. However, others have suggested that active Rac GTPase overexpression is involved in apoptosis in neurons, fibroblasts and epithelial cells [14–19]. Such differential effects of Rac on modulating apoptosis can reflect the cell type- and extracellular stimuli specificity. Therefore, the question as to whether or not the Rac1 protein may be involved in regulating cisplatin-induced apoptosis was directly addressed. Because Rac is a downstream effector of the oncogenic Ras, which is known to participate in carcinogenesis in many human cancers [20], understanding the molecular basis of Rac-modulated apoptosis could lead to strategies to improve the anti-cancer therapeutic benefits.

Cisplatin is one of the most effective and widely used anticancer agents for a variety of human cancers [21]. Cisplatin causes the formation of various reaction products with DNA including covalent DNA adducts and DNA crosslinking, resulting in cell cycle arrest [22], increased DNA repair activity [23–25] and apoptosis [26,27]. Although the detailed mechanisms by which such DNA damage stimulates apoptosis are unclear, recent studies have shown that mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and p38 may be involved in the cellular cytotoxicity in response to cisplatin [28–31].

In this paper, we show that V12-Rac1 prevents cisplatin-induced apoptosis and decreases the p38 MAP kinase activity of NIH3T3 cells stimulated by cisplatin. Furthermore, the p38 MAP kinase inhibitor, and not the JNK and ERK inhibitors, attenuates cisplatin-induced apoptosis.

2. Materials and methods

2.1. Cell lines, antibodies, oligonucleotides and reagents

The NIH3T3 cells were obtained from the ATCC (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Life Technologies, Inc.). The JNK antisense oligonucleotides used in this study were synthesized at ISIS Pharmaceuticals, Inc. (Carlsbad, CA, USA). The oligonucleotide sequences used are as follows: control (ISIS 17552), TCAGTAATAGCCCCACATGG; JNK1 (ISIS 15347), CTCTGTAGGCCCGCTTGG; JNK2 (ISIS 15354), GTCCGGGCCAG-GCCAAAGTC. Cisplatin and propidium iodide (PI) were purchased from Sigma. The MAPK kinase (MEK) (U0126) and p38 inhibitors (SB203580) were obtained from CalBiochem (San Diego, CA, USA). The anti-cytochrome *c* antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). The anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK, anti-ERK and

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Abbreviations: JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase

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anti-phospho-ERK antibodies were all purchased from New England Biolabs, Inc. (Beverly, MA, USA).

2.2. Cell culture

The NIH3T3 mouse embryo fibroblast line was obtained from the ATCC (Manassas, VA, USA) and was maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Life Technologies, Inc.). The cells were cultured at 37°C in a humidified chamber containing 5% CO_2.

2.3. Plasmid constructs

The wild-type *Rac1* cDNA was cloned by reverse transcription-PCR from human Jurkat cells. The dominant positive form of Rac1 (V12-Rac1) was site-directed mutated based on the wild-type *Rac1* cDNA template according to the the manufacturer's instructions (Stratagene, CA, USA). After DNA sequence confirmation, the dominant positive form of *Rac1* cDNA was cloned into a pIND mammalian inducible expression vector driven by a ecdysone-responsive minimal promoter (Invitrogen).

2.4. Transfection and selection

Cells were transfected by the lipofectamine method according to the manufacturer's instructions (Life Technologies, Inc.). Briefly, 1 μg of V12-Rac1-pIND plasmid was incubated with 6 μl of lipofectamine plus reagent at room temperature for 15 min, then 2 μl of lipofectamine reagent added and incubated at room temperature. After 15 min, semiconfluent NIH3T3 cells were washed twice with 1×PBS, then cells were incubated with DNA-lipofectamine-PLUS reagent complexes at 37°C at 5% CO_2 for 4 h. After transfection, the mix was aspirated, and cells were cultured with DMEM medium with 15% FBS for an additional 24 h. Subsequently, cells were incubated with complete medium containing 400 μg/ml of G418 for 5 weeks. Cell clones resistant to G418 were isolated and analyzed. For convenience, one V12-Rac1-transfected cell clone was selected for further studies. Transfected cells were routinely cultured in the presence of 400 μg/ml of G418. However, 2 days prior to and during the experiments, transfected cells were cultured in complete DMEM medium without G418.

2.5. PI staining

The floating and trypsin-detached NIH3T3 cells were collected and washed once with ice-cold PBS and fixed in 70% cold ethanol. After fixation, cells were stained in PBS and PI (50 g/ml), RNase A (100 g/ml), and 0.05% Triton X-100 for 45 min. DNA content of NIH3T3 cells was analyzed by fluorescent-activated cell sorting (FACSort, Becton Dickinson). At least 10 000 events were analyzed, and the percentage of cells in the sub-G1 population was calculated. Aggregates of cell debris at the origin of the histogram were excluded from the analysis of sub-G1 cells, as indicated in the legends to Figs. 2 and 5.

2.6. Preparation of protein lysates and Western blotting analysis

Total cell lysates were prepared on ice with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5% glycerol, 10 mM NaF, 0.3 mM NaMo, 1 mM Na_3VO_4, and 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride). The cell lysates were centrifuged for 30 min at 4°C, and the supernatant was separated. The total protein concentration was measured using a Bio-Rad protein assay kit. 30 μg of total cellular protein was subjected to SDS–PAGE, transferred to nitrocellulose and immunoblotting with either anti-phospho-p38 or anti-p38 antibodies, anti-phospho-JNK or anti-JNK antibodies, and anti-phospho-ERK or anti-ERK antibodies, respectively. The proteins were detected by using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech).

2.7. Release of cytochrome c

The cells were harvested in isotonic mitochondrial buffer (20 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin) and Dounce-homogenized by 20 strokes. The homogenates were centrifuged at 1000×*g* for 10 min at 4°C to eliminate nuclei and unbroken cells. The resulting supernatant was further centrifuged at 15 000×*g* for 20 min at 4°C to obtain the mitochondrial fraction. The supernatant was further centrifuged at 100 000×*g* for 1 h at 4°C to yield the final soluble cytosolic fraction. Cytosolic and mitochondrial proteins were then subjected to Western blot analysis using monoclonal cytochrome *c* antibody (PharMingen).

3. Results and discussion

3.1. Expression of V12-Rac1 prevented cisplatin-induced apoptosis in NIH3T3 cells

To better understand the potential role of Rac1 in cell survival, NIH3T3 cells were stably transfected with Rac1 through the V12-Rac1-pIND plasmid construct under control of the ecdysone-responsive minimal promoter. Following selection by G418 at the concentration of 400 μg/ml for 5 weeks, we isolated nine clones and analyzed V12-Rac1 expression which can be turned on or off by using ponasterone A. Among the nine clones, we have chosen one V12-Rac1 expressing clone (NIH3T3 V12-Rac1), which highly expressed V12-Rac1, for further study in this investigation. Western blot analysis showed that the addition of 5 μM ponasterone A efficiently induced expression of V12-Rac1. Expression of V12-Rac1 became detectable within 12 h of treatment with ponasterone A and the level of Rac1 protein continuously elevated for the duration of ponasterone A treatment (Fig. 1). To investigate whether expression of V12-Rac1 has any effects on cell survival after exposure to cisplatin, cells were treated with or without 5 μM ponasterone A for 24 h and then exposed to 20, 40 or 60 μM cisplatin. After 24 h cisplatin treatment, cells were stained with PI and apoptosis was then measured using FACScan flow cytometry. As shown in Fig. 2, cisplatin caused apoptosis of the V12-Rac1 non-expressing (Pon A (−)) NIH3T3 cells in a dose-dependent manner, with a concentration of 60 μM cisplatin resulting in death of greater than 85% of the cells by 24 h of treatment. However, V12-Rac1 expressing (Pon A (+)) NIH3T3 cells exhibited significant resistance to cisplatin. 24 h after addition of 60 μM cisplatin, less than 35% of cells have undergone apoptosis, which indicated that the dominant active V12-Rac1 protected against cisplatin-induced apoptosis in NIH3T3 cells.

3.2. Role of V12-Rac1 in mediating cytochrome c release in cisplatin-treated cells

Recent studies on apoptosis have shown that cytochrome *c* is released from the mitochondria following the exposure of cells to certain stresses [32]. Therefore, the possibility as to whether or not this pathway is activated by cisplatin was investigated. If it was activated by cisplatin, the possibility of this pathway being inhibited by V12-Rac1 expression was examined. The Pon A (+) and Pon A (−) cells were treated with 60 μM cisplatin for 24 h, after which we isolated mitochondria and cytosolic fraction and performed Western blot analysis using an anti-cytochrome *c* monoclonal antibody to determine the relative content of cytochrome *c* in mitochondria versus the cytoplasm. In the Pon A (−) cells, cytochrome *c* was present at high levels in the cytosolic fraction after exposure to cisplatin (Fig. 3, top panel). However, in the Pon (+) cells, a large amount of cytochrome *c* was present in the mitochondrial fractions after exposure to cisplatin (Fig. 3, bottom panel).

The major pathways of apoptosis have been described in recent years. One pathway is the Fas/APO-1-dependent pathway. Fas/APO-1 interacts with the receptor-associated death protease caspase-8, which eventually activates the downstream effector caspases [33,34]. The second pathway of apoptosis is a

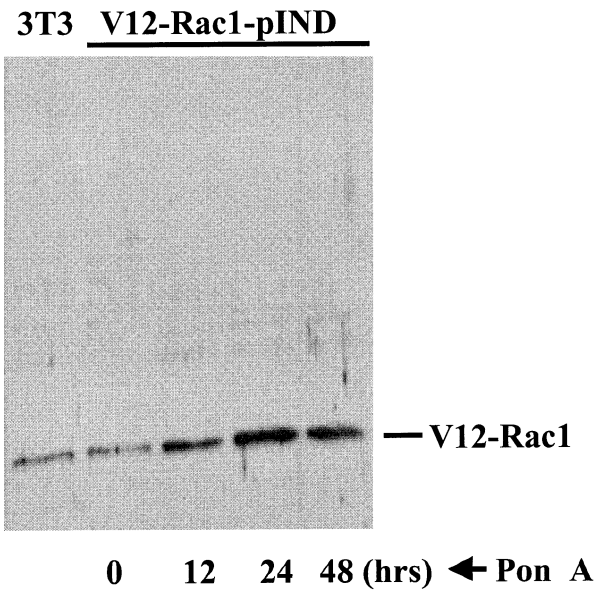

Fig. 1. NIH3T3 cells were transfected with the V12-Rac1 inducible expression vector, V12-Rac1-pIND. Cells were then selected in the presence of G418 as described in Section 2. NIH3T3 V12-Rac1 cells were prepared following ponasterone A (Pon A) treatment for the indicated time. A total of 20 μg cellular protein was separated on 12% SDS–PAGE, then the V12-Rac1 was analyzed, transferred to Hybond ECL membrane, immunoreacted with anti-Rac1 antibody and detected using ECL.

mitochondria-dependent one and results from release of cytochrome *c* leading to caspase-9 activation through the apoptotic protease-activating factor-1 [35]. In this study, increased cytochrome *c* levels were observed in the cytosol after exposure to cisplatin in the Pon A (−) cells. This suggests that cytochrome *c* release plays a role in mediating cisplatin-induced apoptosis in NIH3T3 cells. The ability of V12-Rac1 expression to prevent this effect suggests that V12-Rac1 acts upstream of cytochrome *c* release to exert its survival influence on the cisplatin-treated cells. To identify other factors that may contribute to Rac1-controlled cell survival, the expression levels of a number of signaling molecules that have been implicated in the regulation of cell death were tested. However, no change in either anti-apoptotic (Bcl-2) or pro-apoptotic (Fas, FasL, Bax) molecules was observed in the V12-Rac1 cells as opposed to that observed in the control cells following cisplatin treatment (data not shown).

3.3. *Effect of V12-Rac1 expression on cisplatin-induced MAPKs activation*

To investigate the signaling pathways that mediate cisplatin-induced apoptosis in NIH3T3 cells, and to determine whether V12-Rac1 affects p38, JNK and ERK1/2 MAP kinase activation in response to cisplatin, we examined whether or not p38, JNK and ERK1/2 MAP kinase were activated by cisplatin in the NIH3T3 cells, and whether or not V12-Rac1 modulates the activity of these MAP kinases. NIH3T3 V12-Rac1 cells were treated with or without ponasterone A for 24 h, after which cells were exposed to 60 μM cisplatin for the indicated times and the resulting cell lysates were subjected to Western blot analysis using anti-phospho-p38 antibodies, anti-phospho-JNK polyclonal antibodies and anti-ERK1/2 antibodies to detect phosphorylated p38, JNK and ERK1/2, respectively. As shown in Fig. 4, cisplatin treatment induced strong phosphorylation of p38, JNK and ERK1/2. Exposure of the Pon A (−) cells to cisplatin resulted in an increase in the level of phosphorylated p38 MAP kinase within 2 h with a further increase in the phospho-p38 MAP kinase levels by 24 h (Fig. 4A, top panel). However, treatment of cells with ponasterone A markedly suppressed the cisplatin-induced p38 activation, and p38 activity was first detected 12 h after exposure to cisplatin (Fig. 4A, middle panel). Densitometric analyses showed p38 activation of the Pon A (−) cells at 12 h after cisplatin treatment to be 19-fold higher than p38 activity after treatment with cisplatin for 1 h, whereas peak activation of p38 in Pon A (+) cells at 24 h was only seven-fold higher than at 2 h after cisplatin treatment (Fig. 4A, bottom panel). Cisplatin induced JNK activation of the Pon A (−) cells starting at 8 h, peaking at 12 h and returning to barely detectable levels by 24 h after treatment in the Pon A

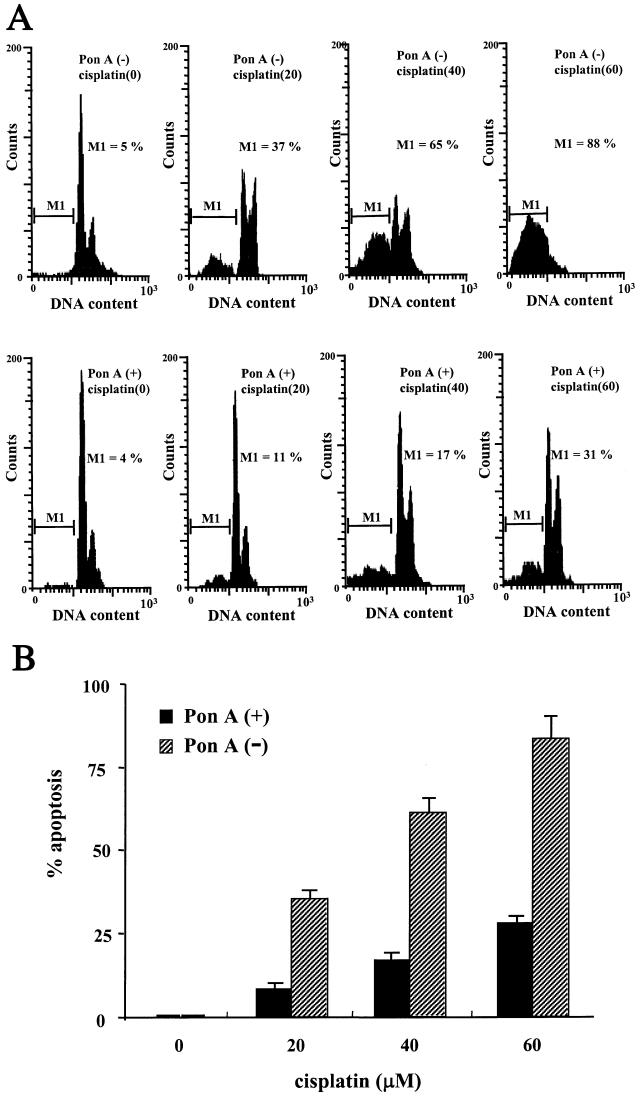

Fig. 2. A: The NIH3T3 V12-Rac1 cells were treated with (+) or without (−) 5 μM ponasterone A (Pon A) for 24 h, after which cells were incubated with different doses of cisplatin for 24 h. The percentage of cells with hypodiploid (apoptotic) DNA content is indicated (M1) as determined by PI staining and flow cytometry. B: Similar results were obtained in three additional experiments. Bars represented S.D. values determined from three independent experiments.

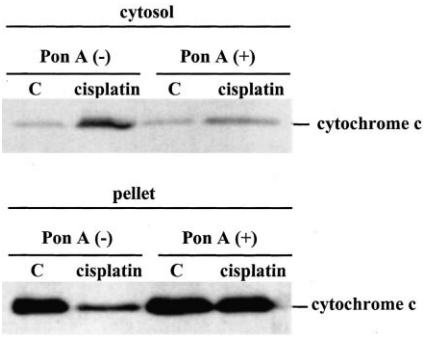

Fig. 3. The NIH3T3 V12-Rac1 cells were treated with (+) or without (−) 5 μM ponasterone A (Pon A) for 24 h, after which cells were incubated with or without (C) 60 μM cisplatin for an additional 24 h. Proteins in cytosolic (top panel) and mitochondrial (bottom panel) fractions of the cells were then subjected to Western blot analysis using antibody against cytochrome *c*.

(−) cells (Fig. 4B, top panel). Cisplatin induced JNK activation of the Pon A (+) cells starting at 12 h and continuing through 24 h after treatment in the Pon A (+) cells (Fig. 4B, middle panel). Densitometry analysis showed peak JNK activation of the Pon A (−) cells at 12 h to be six-fold higher than at 2 h after treatment. JNK activation of the Pon A (+) cells at 24 h after cisplatin treatment is seven-fold higher than JNK activity after treatment with cisplatin for 8 h (Fig. 4C, bottom panel). In contrast to p38 and JNK, V12-Rac1 expression did not affect ERK1/2 phosphorylation over a 24 h period following cisplatin treatment (Fig. 4C). Stripping and reprobing the same blot demonstrated that equal amounts of the p38, JNK and ERK1/2 protein were expressed. Thus, these experiments suggested that p38 and JNK but not ERK1/2 may be involved in the V12-Rac1-mediated cell survival after exposure to cisplatin in NIH3T3 cells.

3.4. p38 but not JNK and ERK1/2 are involved in cisplatin-induced apoptosis

Cellular responses to many external stimuli involve the activation of several types of MAPK signaling pathways including ERK1/2, JNK, and p38 kinase. The ERK1/2 pathway is regulated mostly by mitogenic stimuli, and leads to the production of proteins required for cell growth and differentiation [36]. In contrast, JNK and p38 are activated primarily by various stresses and are involved in cell transformation, stress responses and apoptosis [37]. Because the V12-Rac1 expression decreased the p38 MAP kinase activity and delayed increase of JNK in phosphorylation after exposure to cisplatin in NIH3T3 cells, we next wanted to examine whether these two MAP kinases are participating in the cisplatin-induced apoptosis in NIH3T3 cells.

To address the potential involvement of p38 in cisplatin-

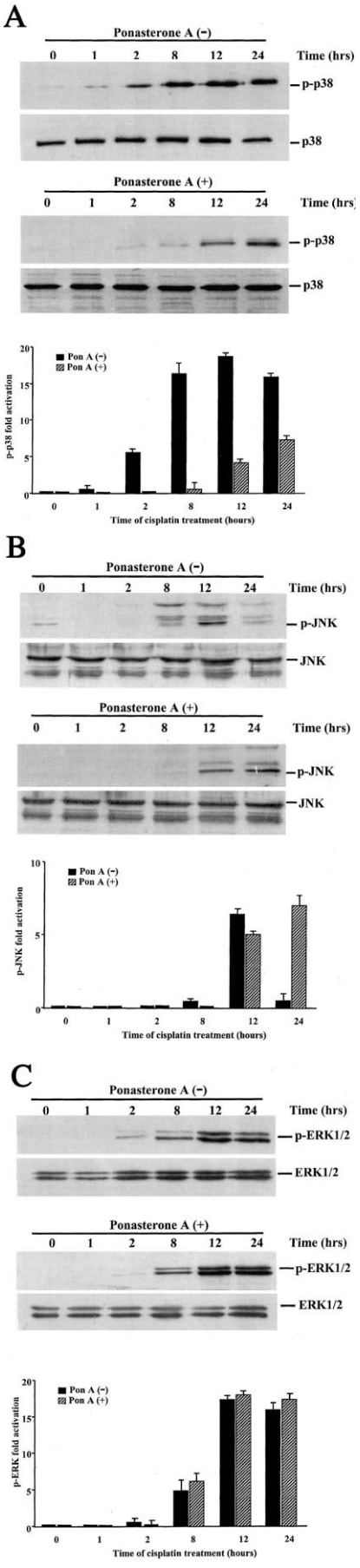

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Fig. 4. The NIH3T3 V12-Rac1 cells were treated with (+) or without (−) 5 μM ponasterone A (Pon A) for 24 h, after which cells were incubated with 40 μM cisplatin for the indicated times. p38 (A), JNK (B) and ERK1/2 (C) activation were determined by Western blot analysis using antibodies recognizing the phosphorylated form of ERK1/2, p38 and JNK. Total ERK1/2, p38 and JNK protein levels were detected by Western blot analysis using anti-ERK1/2, anti-p38 and anti-JNK antibodies, respectively. Fold activation was determined for p-p38, p-ERK and p-JNK by scanning the bands for densitometric analysis.

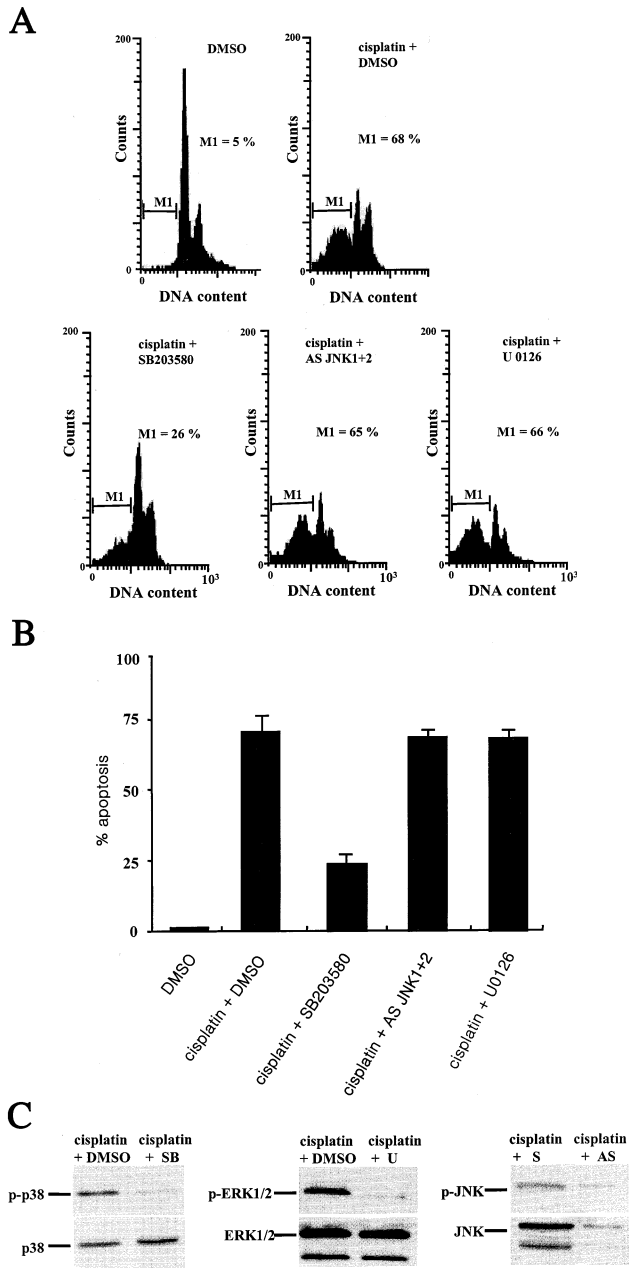

Fig. 5. A: NIH3T3 cells were pretreated with vehicle (DMSO), 10 μM SB203580 or 20 μM U0126 for 30 min followed by incubation with or without 40 μM cisplatin. NIH3T3 cells transfected with 0.2 μM antisense JNK1 plus 0.2 μM antisense JNK2 (AS JNK1+2) were treated with 40 μM cisplatin. After 24 h cisplatin treatment, the cells were stained with PI, after which apoptosis was analyzed by flow cytometry. B: Similar results were obtained in three additional experiments. Bars represent S.D. values determined from three independent experiments. C: Inhibition of cisplatin-induced p38 and ERK1/2 phosphorylation by the p38 inhibitor SB203580 (SB: 10 μM) and the MEK1/2 inhibitor U0126 (U: 20 μM), respectively. Attenuation of JNK protein levels by JNK1+2 antisense oligonucleotides (AS), but not by sense oligonucleotide (S). p38, ERK1/2 and JNK activities were carried out as in Fig. 4.

induced apoptosis in NIH3T3 cells, the cells were pretreated with p38 specific inhibitor, SB203580 (10 μM), for 30 min. Cells were then treated with 40 μM cisplatin for an additional 24 h, after which cells were stained with PI, and apoptosis was then measured using FACScan flow cytometry. As shown in Fig. 5A,B, compared with the untreated cells, pretreatment with 10 μM SB203580 resulted in a significantly lower apoptotic cell population. We observed that pretreatment of NIH3T3 cells with 10 μM SB20358 totally abolished p38 phosphorylation induced by cisplatin treatment (Fig. 5C, left panel). To test that SB203580 was not inhibiting JNK activity as well, we measured JNK activity and found that SB203580 had little effect on cisplatin-induced JNK activation (data not show). Therefore, these results suggest that the induction of p38 activity is required, at least in part, for the cisplatin-induced apoptosis.

To investigate the effect of JNK on the cisplatin-induced apoptosis, we conducted functional knock experiments using antisense oligonucleotides to JNK1 (ISIS 15347) and JNK2 (ISIS 15354), which were phosphorothioate oligonucleotides targeted to JNK1 and JNK2 mRNA to block the JNK/SPAK pathway [38]. Repeat analyses of antisense oligonucleotide functional knock of JNK were performed for comparative purposes. NIH3T3 cells transiently transfected with either 0.2 μM each antisense JNK1 and JNK2 oligonucleotides (JNK1+2AS) were cultured with 40 μM cisplatin for 24 h. Antisense oligomers to JNK1/2 had no protective effect on the cisplatin-induced apoptosis (Fig. 5A,B), suggesting that JNK activity is not involved in the cisplatin-induced apoptosis in NIH3T3 cells. Evidence for transfection efficiency and functionality of the antisense oligonucleotide to JNK1/2, in comparison to scrambled oligonucleotides (ISIS 17552), exhibited significantly reduced expression of JNK (Fig. 5C, right panel). We also examined the role of ERK1/2 activation in the cisplatin-induced apoptosis. As we expected, the ERK1/2 inhibitor U0126 had no effect on cell survival (Fig. 5).

Cisplatin-induced apoptosis in NIH3T3 cells is potently inhibited by p38, probably acting via cytochrome *c* release from the mitochondria. Therefore, the possibility as to whether or not p38 activation exerted an effect on cisplatin-induced cytochrome *c* release from mitochondria was examined. As shown in Fig. 6, 10 μM SB203580 efficiently inhibited cytochrome *c* release from the mitochondria following cisplatin treatment. This implies that p38 activation is involved in the release of cytochrome *c* during cisplatin-induced apoptosis.

p38 is associated with induction of apoptosis in numerous cell types in response to different cellular stresses [39–41]. However, p38 activation may either prevent cell death or trigger apoptosis depending upon the cell type and specific death stimulus [42]. Recently, two groups have reported that p38 activation following cisplatin treatment is involved in cisplatin-induced apoptosis [43,44] However, others have shown that phosphorylated p38 has no effect on cisplatin-induced apoptosis [45,46]. Such varying results may depend on the cell type specificity. However, in this study using NIH3T3 cells, the p38 MAP kinase inhibitor, SB203580, was quite

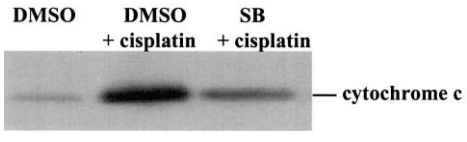

Fig. 6. NIH3T3 cells were pretreated with vehicle (DMSO) or 10 μM SB203580 (SB) for 30 min and cells were then treated with or without 60 μM cisplatin. After 24 h, the cytosolic preparations were tested for the accumulation of cytochrome *c* by Western blot analysis, using a monoclonal antibody against cytochrome *c*.

effective in protecting NIH3T3 cells against cisplatin-mediated apoptosis but not the JNK and ERK inhibitors.

Our experiments demonstrated that cisplatin treatment led to apoptosis, which was associated with release of cytochrome *c* from the mitochondria, and V12-Rac1 could inhibit cisplatin-induced apoptosis in the NIH3T3 cells. In addition, dominant active V12-Rac1 attenuated p38 activity but not JNK and ERK1/2 after exposure to cisplatin. Moreover, the p38 MAP kinase inhibitor, SB203580, was quite effective in protecting NIH3T3 cells against cisplatin-mediated apoptosis. The correlation of induced dominant active V12-Rac1 expression with increased cell survival as well as decreased p38 activity after exposure to cisplatin suggests that raising the level of V12-Rac1 may prevent cisplatin-induced apoptosis by suppressing p38 activation. Further studies to identify the downstream target of the Rac cascade in the cisplatin signaling pathway are recommended. Characterization of this pathway will contribute to the understanding of how dominant active Rac1 has a pro-survival role in cisplatin-induced apoptosis.

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