Lack of c-Jun activity increases survival to cisplatin

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Abstract Antineoplasic agents such as cisplatin and adriamycin execute their pharmacological role by triggering apoptosis. We have studied the mechanism of apoptosis induction by cisplatin and adriamycin. Both drugs activated JNK with slow and persistent kinetics. Adriamycin activated caspase-3 before the rise in JNK activity, while the response to cisplatin occurs hours after JNK activation. The increase in JNK activity was necessary for cisplatin-mediated apoptosis but it was dispensable for adriamycin-induced cell death. Cells derived from c-jun knock out mice were more resistant to cisplatin cell death than normal cells, but no difference was observed in response to adriamycin. Activation of JNK and cell death by cisplatin is mediated by the MEKK1/SEK1 cascade, since expression of dominant negative expression vectors of these kinases blocked both processes. p38 was also activated by cisplatin with similar kinetics as JNK. AP-1 complexes were activated by cisplatin including mainly c-jun/ATF-2 heterodimers suggesting that AP-1-dependent transcription partially mediated cisplatin-induced apoptosis.

Key words: Cisplatin; JNK1; jun; CPP32; DNA damage; Apoptosis

1. Introduction

Antineoplasic agents such as cisplatin, etoposide, adriamycin and taxol carry out their pharmacological role by eliciting the apoptosis program in the cells. Recent evidence indicates that the principal effectors of apoptosis are a series of proteases called caspases which inactivate proteins involved in DNA repair and apoptosis protection [1] and activate proteins involved in apoptosis induction [2]. Most antineoplasic agents also activate different kinase cascades, such as the stress-activated protein kinase JNK/SAPK [3] that phosphorylates the c-jun transcription factor at Ser-63 and Ser-73 [4] within its transactivation domain [4–6]. The JNK/SAPK has also been shown to phosphorylate and regulate the activity of other transcription factors including ELK-1 and ATF-2 [4,5,7–9]. JNK/SAPK is a component of a sequential protein kinase pathway [3,5,7,10–14] that similar to the ERK members of the MAPK family requires phosphorylation for its enzymatic activation. Its phosphorylation state is positively controlled by JNK kinase (JNKK/stress-activated ERK kinase, SEK1) [3,10,15,16]. JNKK/SEK1 is itself regulated by phosphorylation by an upstream kinase referred to as MEK kinase (MEKK) [14] or alternatively ASK-1 [17]. A third MAPK isoform is p38, that is activated in response to cellular stress and osmotic shock by phosphorylation in a Tyr and Thr residue and phosphorylates, among others, the ATF-2 transcription factor [10,12,18].

c-jun forms, together with the Fos family of proteins, the AP-1 transcription factor which has been implicated in a number of biological processes [19]. c-Jun-related proteins, JunB and JunD, can also function as transcription factors either as homodimers or bound to partner proteins, such as ATF-2, CREB or Fos-related proteins [20,21]. c-Jun is rapidly and transiently activated in response to mitogens, indicating that c-Jun probably has a function in the control of cell proliferation [22–25]. In agreement with this hypothesis, cells lacking a functional c-jun allele grow slower in vivo and in vitro [26–28]. On the other hand, an increased c-Jun activity is able to induce apoptosis in immortalized NIH3T3 fibroblasts [29].

The consequences of JNK activation on cell fate seem to be dependent on the type of stimuli. In cells treated with UVC [30], JNK activation seems to have a protective effect and in response to other stimuli, such as TNF-α [31] or CD45 [32], it does not play a role in apoptosis. On the contrary, in cells committed to apoptosis by treatment with antineoplasic agents, such as cisplatin, vincristin or adriamycin, JNK is also activated, although its role on apoptosis is not well-established. We show in this article that although both cisplatin and adriamycin activate JNK and caspases, the final role of JNK on the cell fate is different. Whilst in cells treated with cisplatin, activation of the JNK pathway is necessary for caspase activation and apoptosis induction, in cells treated with adriamycin, activation of JNK occurs after caspase activation and it is dispensable for apoptosis induction. These differences could have important implications in the therapeutic application of both compounds.

2. Materials and methods

2.1. Cell lines, antibodies, plasmids and reagents

The epidermal keratinocyte cell line Pam 212 and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and supplemented with 10% fetal calf serum and 1 mM L-glutamine. NIH3T3 cells derived from c-jun knock out mice were cultured in the same medium but supplemented with 5% fetal calf serum. Rabbit anti-JNK1 serum (anti-C-terminal domain), anti-p38 (AA 341-360), anti-c-Jun (C-terminal domain), anti-c-Fos (amino-terminal domain), anti-ATF-2 (carboxi-terminal domain) and anti-PARP (N-terminal) were from Santa Cruz Biotechnology. Plasmids pMEKK1, pMEKK1(K-R), pSEK1 and pSEK1(K-R) were obtained from Michael Karin [33]. NIH3T3 cells derived from knock out mice for c-jun were obtained from Erwin Wagner [27,34]. Jun−/+ cells were obtained by co-transfecting Jun−/− cells with the vector pCDNAI-c-jun and the p-PUR plasmid (Clontech) by the calcium phosphate method and selecting stable transfectants by puromycin resistance.

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2.2. Cell treatments

Cells were cultured at a density of 10^6 cells by a 100 mm plate and subjected to different treatments. For the UV irradiation, cells were shifted into medium containing 0.5% FBS during 24 h, the medium was then removed and cells were irradiated with 40 J/m^2 by using a UV germicidal lamp. For cisplatin and adriamycin (Sigma) treatments, after culturing during 24 h in 0.5% FBS, cells were incubated with the drugs during the periods of time indicated in each experiment.

2.3. Transient transfections and β-galactosidase assays

Cells were plated 24 h before transfection at a density of 3×10^5 cells/60 mM plate as previously described [35]. Cells were co-transfected with the plasmids pCMVβ-galactosidase and plasmids encoding the dominant negative mutants MEKK1-KR and SEK1-KR. For kinase assays, cells were incubated in 0.5% FCS during 24 h and treated with cisplatin for 6 h and the efficiency of transfection was estimated by assaying for β-galactosidase activity. For the death assay, cells were transfected as before and allowed to recover during 8–10 h in complete medium and then treated with cisplatin. Cells were harvested at 20 h after treatment, washed and fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS) and then washed twice. Fixed cells were then incubated in reaction solution (PBS pH 7.4, 1 mM $MgCl_2$, 10 mM $K_4(Fe(CN)_4)$, 10 mM $K_3(Fe(CN)_4)$ and 1 mM X-gal) for 1 h and washed twice with PBS. Apoptotic cells were identified by their shrunk morphology and intense staining. The person determining the percentage of apoptotic cells was not aware of the treatment.

2.4. Extract preparation and solid phase kinase assays

Whole cell extract preparations for JNK assays were carried out as described [36]. 20–40 μg of extract was incubated with GST-c-jun-79 fusion protein bound to agarose beads and incubated in the presence of 1 μCi [γ-^{32}P]ATP in 30 μl kinase buffer. The kinase reaction was performed at 30°C for 30 min and stopped with one volume of SDS sample buffer. For the p38 MAPK assay, cells were lysed as described [36] and p38 MAPK was immunoprecipitated with specific antibody/protein A-Sepharose (Pharmacia). Immunoprecipitates were subjected to in vitro kinase assays by using purified GST-ATF-2 as the substrate. Reactions were carried out during 30 min at 30°C. The reaction mixtures were resolved by SDS-PAGE analysis. The amount of radioactivity incorporated in each lane was determined with an instantimager (Packard) and corrected against the amount of peptide in each lane as determined by gel staining with Coomassie blue.

2.5. Cell viability assay

The cell viability was determined by using a crystal violet staining method as previously described [36]. For propidium iodide staining, cells were treated as indicated [36], propidium iodide was added at a concentration of 10 μM during 10 min and the cells were directly visualized under a Zeiss microscope at a 630-fold magnification.

2.6. Western blot analysis

30 μg of total cellular protein was subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with a 1:1000 dilution of either anti-JNK1, anti c-Jun or anti-PARP antibodies. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham) using a biotinylated anti-rabbit antibody and peroxidase-coupled streptavidin.

2.7. Gel retardation analysis

Cells were serum depleted during 24 h and whole cell extracts obtained as described [20]. Gel retardation analysis was performed as described [37] using double-stranded oligonucleotides containing the sequences 5′-AGCTGGGGTGACATCATGGG-3′ (JUN1: c-jun nucleotides −75–−60), 5′-AGCTAGCATTACCTCATCCC-3′ (JUN2: c-jun nucleotides −194–−179). 5 μg of whole cell extract was incubated with 5×10^4 cpm of ^{32}P-labelled probe for 30 min at 4°C and the complexes were separated in a 4% polyacrylamide gel in 0.5×Tris-borate-EDTA buffer. To test the effect of specific antibodies, 1 μl antiserum was added to the incubation mixture for 30 min at 4°C prior to the addition of labelled probe. The binding specificity was confirmed by competition with a 100-fold excess of non-labelled JUN1, JUN2 or SP1 (5′-GATGTGTGGGAGGAGCTTCT-3′) oligonucleotides.

3. Results

3.1. c-jun contributes to cisplatin- but not adriamycin-induced apoptosis

c-jun is phosphorylated in response to activation of JNK by antineoplasic drugs, such as cisplatin [36] and adriamycin [38]. In order to elucidate if activation of the JNK pathway is necessary for apoptosis induction by these drugs, we have used immortalized 3T3 fibroblasts derived from mouse embryos without c-jun, due to targeted disruption of the c-jun gene by homologous recombination [27]. This cell line shows an increased sensitivity to certain types of stress such as UVC radiation [34]. We first examined whether the absence of c-jun affects the cell survival after exposure to different doses of cisplatin. As it can be observed in Fig. 1a, cells lacking c-jun were more resistant to cisplatin than parental cells. The ID_{50} for jun−/− cells was 10-fold higher than that obtained with the wild-type cells. The increased viability observed in jun−/− cells could be due to an unspecific clonal

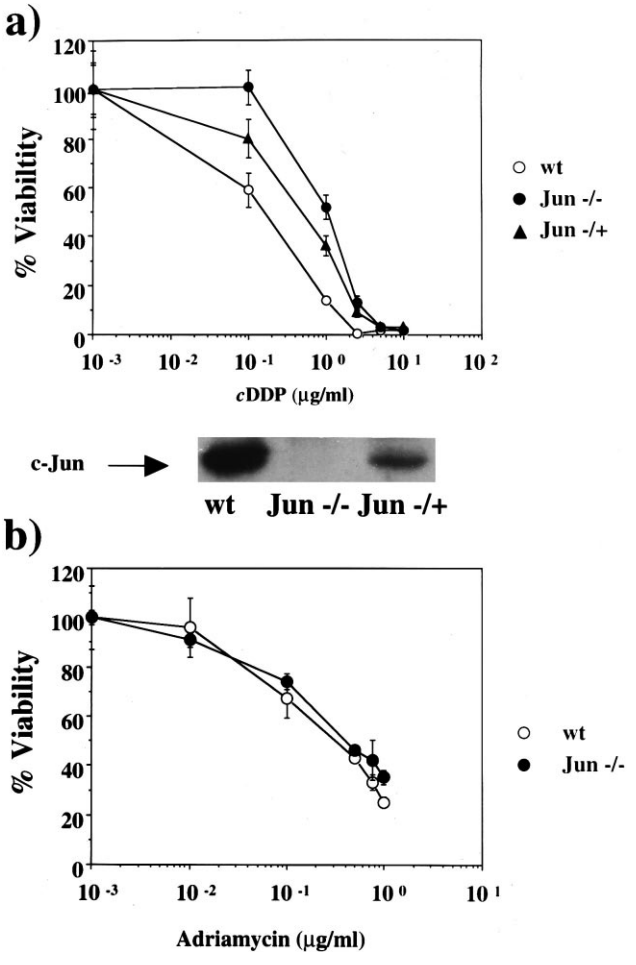

Fig. 1. Cell viability after cisplatin and adriamycin treatment of cells lacking the c-jun gene. NIH3T3 cells derived from control mice (WT), knock out mice for c-jun (jun−/−) or jun−/− cells transfected with c-jun (jun−/+) were treated at different concentrations of cisplatin (a) or adriamycin (b) for 48 h and the viability was estimated by a crystal violet-based staining method. Inserted is a Western blot showing the expression levels of c-jun in each cell line. The data represent the means of a single experiment performed in triplicate. The experiment was repeated twice with similar results.

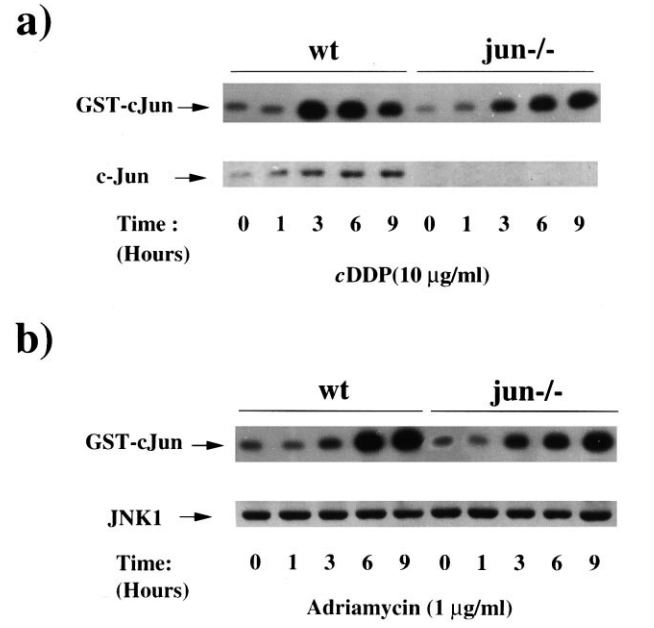

Fig. 2. JNK activation in response to cisplatin and adriamycin. NIH-derived cell lines from control animals (WT) or derived from jun−/− animals were serum-depleted during 24 h and treated with (a) cisplatin (10 μg/ml) or (b) adriamycin (1 μg/ml). Cell lysates were used for determination of the JNK activity (upper panels) by the agarose-coupled GST-c-Jun kinase assay or subjected to a Western blot (lower panels) for analysis of (a) c-jun or (b) JNK expression. The experiment was repeated twice with similar results.

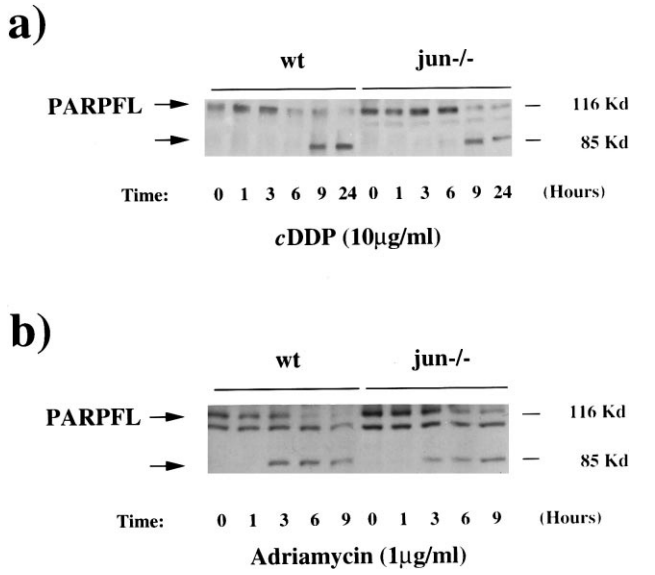

Fig. 3. Caspase-3 activity in cisplatin- and adriamycin-treated cells. Control WT or Jun−/− cells were treated with (a) cisplatin (10 μg/ml) or (b) adriamycin (1 μg/ml). Extracts were prepared at different times after treatment and a Western blot was performed with a polyclonal antibody directed against PARP. The full length protein (116 kDa) is designed as PARPFL. The 85 kDa proteolytic fragment is indicated with an arrow.

effect of this cell line. To check this possibility, we have generated the cell line jun−/+, by transfecting the c-jun gene into the jun−/− cell line (Fig. 1a). Although Jun−/+ cells expressed lower levels of c-jun than the wild-type, as determined by a Western blot analysis, the sensitivity to cisplatin is higher than that of the jun−/− cell line, indicating that a lack of c-jun was indeed the cause of the increased viability to cisplatin of the jun−/− cells. On the contrary, when we tested the sensitivity of jun−/− or parental cells to adriamycin, we observed no detectable differences. We then studied the activation of JNK of both cell lines to cisplatin and adriamycin. As shown in Fig. 2, both compounds were able to activate JNK in both cell lines in a similar fashion and intensity. The only difference observed in the parental cell was a delayed JNK activation in response to adriamycin, reaching the maximal activation at 6 h after drug addition, in contrast to cisplatin that reached the peak at 3 h.

3.2. *Caspase-3/CPP32 activity is not required for activation of the JNK pathway by cisplatin but is necessary for apoptosis induction*

Increasing evidence in the literature indicates that the proteolytic cleavage of critical subcellular substrates by the caspase family of proteases [39–41] plays an important role in the executionary phase of the apoptotic process [42]. This family of proteinases cleaves their target proteins behind an aspartic acid [42–45] in a specific sequence. During apoptosis, a discrete set of cellular proteins becomes cleaved by ICE/CED-3-related proteinases, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), protein kinase Cδ(PKCδ) and others (reviewed in [39]). In order to investigate the difference observed in sensitivity of jun−/−

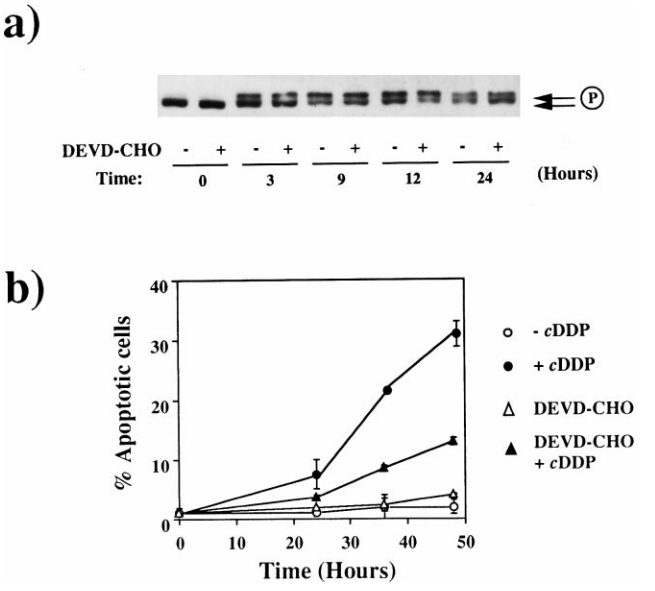

Fig. 4. Caspase-3 activity is not necessary for JNK activation by cisplatin. (a) JNK activity after caspase-3 inhibition. Pam 212 cells were serum-harvested during 24 h and treated with 10 μg/ml cisplatin. When indicated, cells were pretreated with DEVD-CHO (100 μM). At different time points, cells were collected and the lysate was assayed for JNK expression and activation by Western immunoblotting using a polyclonal antiserum against JNK1. The arrows indicate the unphosphorylated or phosphorylated (P) forms of JNK1. The experiments were repeated twice with similar results. (b) Apoptosis detection by propidium iodide staining. Pam 212 cells were stimulated with cisplatin after pretreatment with DEVD (1 h at 100 μM) in normal medium or not and stained with propidium iodide. The apoptotic cells were quantified by fluorescence microscopy.

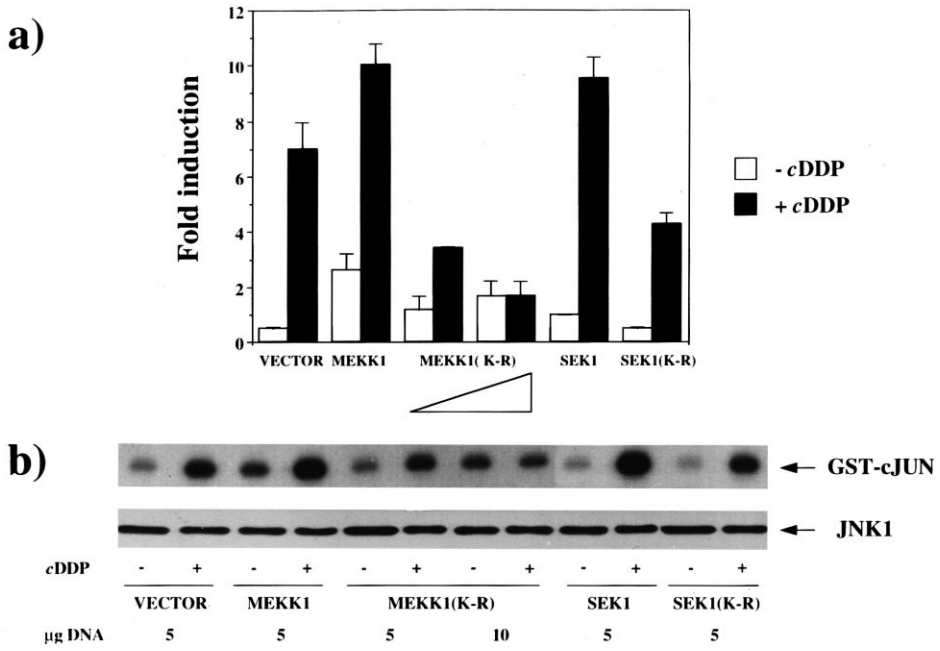

Fig. 5. Cisplatin requires MEKK1 for activation of JNK. 293T cells were transiently transfected with the indicated amounts of DNA encoding the wild-type or the dominant negative forms of MEKK1 or SEK1 kinases. Cells were then serum-depleted during 24 h and treated with cisplatin (10 μg/ml) during 6 h. The transfection efficiency was monitored by transfecting in parallel a plate with pCMVβ-galactosidase which was stained for enzymatic activity. Endogenous JNK was measured in cell extracts by using agarose beads coupled to purified GST-c-jun protein. (a) The quantification in an instantimager of a typical experiment where the amount of the radioactivity incorporated in each sample was related to the vector-untreated sample, considered as one. (b) Autoradiogram of the samples quantified in a. In the lower panel, a Western blot of JNK of the samples. The data represent the means of a single experiment performed in triplicate. The experiment was repeated twice with similar results.

cells to cisplatin in contrast to adriamycin, we studied the timing of caspase activation in response to both drugs. Cleavage of PARP was used as an assay for caspase-3 (CPP32) activity. Both parental and jun−/− cells responded to cisplatin and adriamycin by cleaving PARP (Fig. 3). However, we observed significant differences in the kinetics of caspase-3 activation in response to both drugs. While activation induced by cisplatin (Fig. 3a) was detected 9 h after drug addition, adriamycin activates caspase-3 3 h after treatment. Similar results were obtained by using a specific substrate for CPP32, DEVD-AMC (data not shown).

Recently, it has been demonstrated that MEKK1 is a substrate for caspase-3, that cleaves this protein at Asp-874 [2]. Proteolytic cleavage at this site generates a COOH-terminal fragment encoding the MEKK1 kinase domain [46]. Previous reports indicated that genotoxins, such as UVC induce proteolytic degradation of MEKK1, a few hours after DNA damage [47]. We then tested if activation of JNK by both drugs could be influenced by the activity of caspase-3. As shown in Fig. 4a, treatment of Pam 212 cells with cisplatin alone or after 1 h pretreatment with DEVD-CHO (an inhibitor of caspase-3) showed no detectable inhibition in JNK activation at any time after treatment. Similar results were obtained with adriamycin, in agreement with previous results [48]. By contrast, activation of apoptosis by cisplatin (Fig. 4b) and adriamycin (not shown) could be inhibited by DEVD-CHO. As caspase-3 is activated in response to adriamycin before JNK is activated, the results suggest that activation of JNK in response to this drug is not necessary for apoptosis induction.

3.3. Activation of JNK and cell death by cisplatin is dependent on the MEKK1 and SEK1 activities

Since c-jun seems to participate in apoptosis induction by cisplatin, we studied the pathway involved in JNK activation by this drug. Activation of JNK in response to several agents is elicited through activation of a cascade of kinases [49] that includes MEKK1/SEK1. We have used transient transfection assays in 293T cells in order to examine the effect of expression of the dominant negative mutants for MEKK1(KR) and SEK1(KR) on JNK activation in response to cisplatin (Fig. 5). Although expression of MEKK1 or SEK1 slightly enhanced JNK activation in response to cisplatin, expression of either MEKK1(KR) or SEK1 (KR) prevented in a dose-dependent manner JNK activation by this drug without affecting the levels of JNK protein (Fig. 5). In conclusion, the results indicate that activation of JNK by cisplatin involves and requires the MEKK1-SEK1 pathway.

We then tested if interfering with the MEKK1/SEK1/JNK pathway had a suppressive effect on apoptosis. 293T or Pam 212 cells were transfected with the pCMVβ-galactosidase plasmid alone or together with plasmids encoding the dominant negative kinase mutants and the cells were treated with cisplatin. After 20 h, the cells were harvested and stained with X-gal to analyze the β-galactosidase expressing cells (blue color). The survival rate of transfected cells after drug treatment was estimated by monitoring the cell morphology among the blue cells. Cells were assigned as apoptotic when they were exhibiting a shrunk cell shape. The dominant negative mutants of MEKK1 and SEK1 enhanced cell survival of transfected Pam 212 (Fig. 6a) or 293T cells (not shown). In both cell lines,

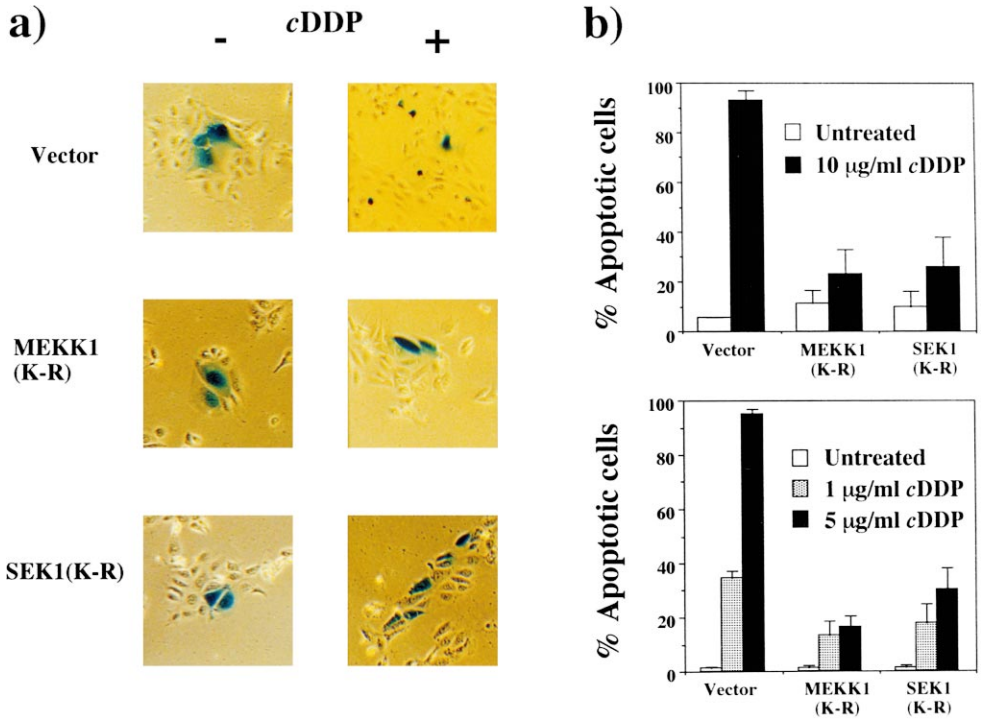

Fig. 6. Apoptosis induced by cisplatin involves activation of the MEKK1/SEK1 pathway. Pam 212 and 293T cells were transiently transfected with 5 μg of the indicated plasmid and 1 μg of pCMV-β-galactosidase. Transfected cells were incubated in complete medium, treated with the indicated concentrations of cisplatin and processed for β-galactosidase activity. In (a), Pam 212 cells were transfected and the apoptotic cells were identified as indicated in Section 2. (b) Quantification of experiments described in (a), performed in Pam 212 (upper panel) or 293T cells (lower panel). Data are reported as the percentage of apoptotic cells among the total blue cells. An average of 400 cells were examined in different fields for each plate. The data represent the means of a single experiment performed in triplicate. The experiments were repeated twice and similar results were obtained.

apoptosis was reduced by more than 50% in cells transfected with the kinase defective mutants (Fig. 6b). These results indicate that interfering with the JNK pathway partially prevents mortality induced by cisplatin, suggesting that this pathway contributes to cell death.

3.4. *AP-1 complexes induced by cisplatin contain c-Fos, c-Jun and ATF-2 proteins*

Our results indicated that c-Jun protein levels are increased in cisplatin-treated cells (Fig. 2). Transfection of an expression vector encoding a dominant negative c-jun mutant Flag Δ169 [50] resulted in protection to apoptosis induction by cisplatin (data not shown). These results are consistent with the idea that phosphorylation of the c-Jun protein at its N-terminal transactivation domain by JNK is one of the events involved in apoptosis induction, since its deletion partially protects cells to cisplatin. These results suggested that de novo transcription is required for this process. In agreement with these hypothesis, treatment of Pam 212 cells with actinomicin D, an inhibitor of RNA synthesis, partially protected them from death induced by cisplatin treatment (data not shown), suggesting that cisplatin induces transcription of genes that could be involved in apoptosis.

Since activation of c-Jun is required for apoptosis, we wish to elucidate which types of AP-1 complexes were induced upon cisplatin treatment. We have used the AP-1 sites found at the c-jun promoter [37,46] for this study. Both sites, named JUN1 and JUN2, have been found to contribute to UV-mediated induction of c-jun transcription in several cell types [37]. Extracts from Pam 212 cells treated with cisplatin (Fig. 7) showed a transient increase (3 h) in the amount of AP-1 complexes, bound to the JUN1 oligo. To identify the nature of factors bound to this site, we pre-incubated nuclear extracts with specific antibodies against c-Jun, c-Fos or ATF-2. The results indicated that the complexes were mainly composed from c-Jun and c-Fos. We have also used extracts from cells irradiated with UVC (40 J/m^2) and also detected a transient increase in the amount of these complexes although at shorter intervals after treatment (1 h). A different kinetic was obtained when we used an oligo corresponding to the JUN2 site. Cisplatin induced an increase in the amount of these complexes that is permanent up to 9 h of treatment and contained mainly c-Jun and ATF-2 proteins (Fig. 7a, b). As observed with the JUN1 site, the increase in the amount of complexes bound to this oligo was transient when we treated the cells with UVC. These results are in agreement with previous results indicating that cisplatin induces an increase in c-Jun protein that persists during at least 12 h after drug addition. Additionally, we have observed an increase in p38 activity that showed the same kinetic of activation as JNK in response to cisplatin (Fig. 7c). The parallel increase in both activities could, therefore, explain the continuous increase in AP-1 complexes containing c-Jun and ATF-2. AP-1 complexes are involved in upregulation of the c-jun promoter [37] but in addition, these complexes could be necessary for the induction of de novo transcription of genes involved in cell death.

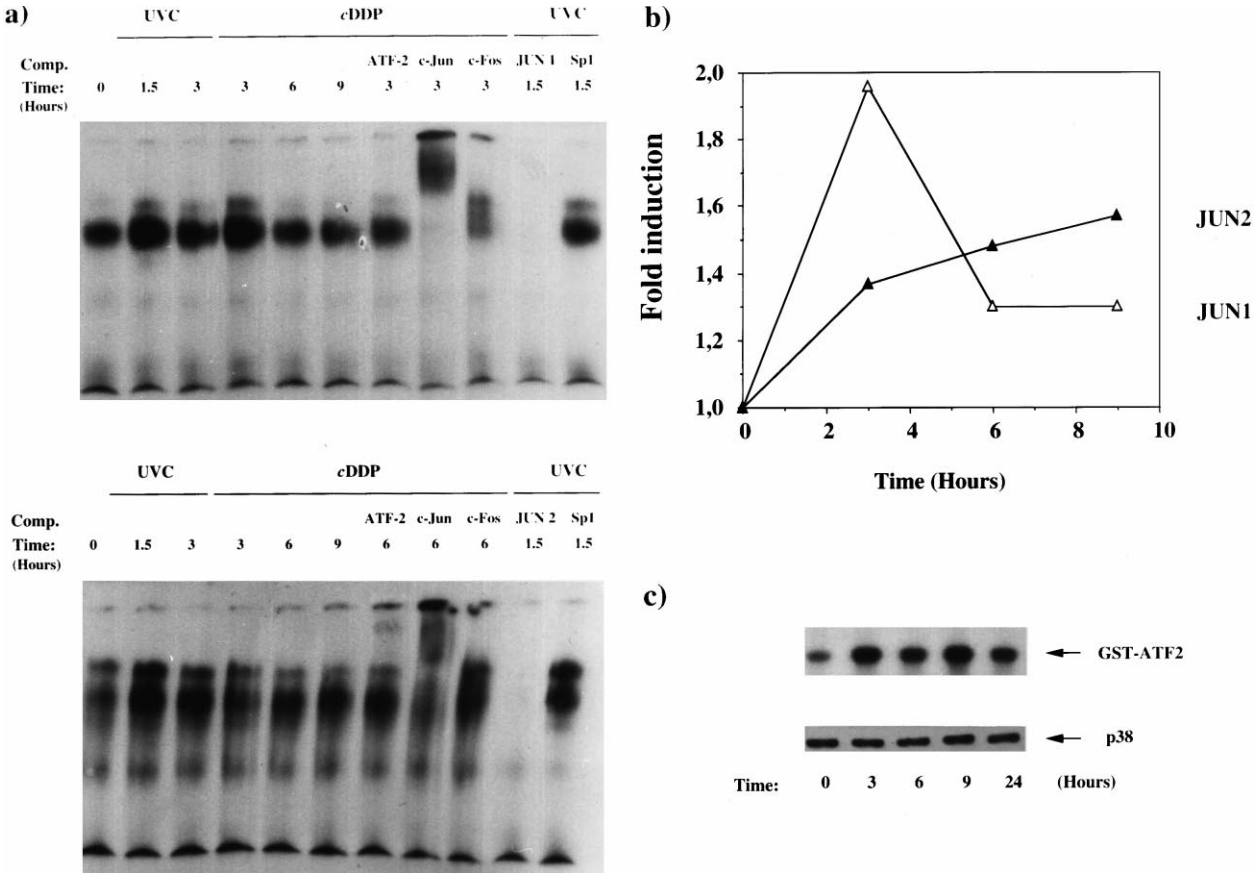

Fig. 7. Analysis of AP-1 complexes induced by cisplatin. Pam 212 cells were serum-depleted during 24 h and treated either with 10 μg/ml cisplatin or irradiated with 40 J/m^2 and harvested at the indicated times. Total extracts were obtained and incubated with ^{32}P-labelled oligonucleotides corresponding to the JUN1 (upper panel) or JUN2 (lower panel) AP-1 sites of the c-jun promoter. When indicated (Comp.), samples from cells treated during 3 h (upper) or 6 h (lower) with cisplatin were incubated with antibodies specific for ATF-2, c-Jun or c-Fos. Extracts from UV-treated cells were subjected to competition with oligonucleotides containing either JUN1, JUN2 or Sp1 sites. (b) DNA-bound complexes from cisplatin-treated cells specific to JUN1 and JUN2 sites were quantified and represented in relation with untreated cells. The experiments were repeated twice with similar results.

4. Discussion

Pathological disturbance of the balance among cell growth and apoptosis often leads to an imbalance in the cell number and could lead to tumorigenesis [51]. Thus, manipulation of the signal pathways that control apoptosis has emerged as an important strategy for anti-cancer therapy. Additionally, several anti-cancer drugs (cisplatin, etoposide, adriamycin) exert their therapeutical action by inducing apoptosis in the treated cells. Therefore, determining the intracellular pathways activated by these drugs could improve clinical protocols. In these studies, we present evidence demonstrating that interfering with the SAPK pathway and the transcriptional activity of the c-Jun protein increased the survival to cisplatin.

Our results support evidence from other laboratories indicating that the role of the JNK pathway in apoptosis depends on the genotoxic agent. By using two different anti-neoplasic drugs that activate JNK, we have found that this enzyme is an important mediator of apoptosis induced by cisplatin but not by adriamycin. We observed that mouse fibroblasts lacking the c-jun gene were more resistant to cisplatin than normal cells, while jun−/+ cells recover sensitivity to cisplatin. By contrast, no difference was found when the cells were treated with adriamycin similarly to the results observed with other DNA damaging agents such as UVC [37]. Our results suggest that in cisplatin-treated cells, induction of apoptosis can take place as a consequence of JNK activation. In cells treated with adriamycin, caspases are probably activated throughout a different pathway that does not require JNK activation as described for Fas-induced apoptosis [32].

We have observed that not only JNK but also p38 MAPK is activated in cells treated with cisplatin. Activation of both cell signaling cascades has been involved in response to other DNA damaging agents, such as UVC, MMS and gamma radiation [52]. Our results showed that activation of JNK in response to cisplatin involves the activity of the sequential MEKK1 and SEK1/MEKK4 kinase cascade. Activation of this cascade by cisplatin takes place in the absence of other survival pathways, such as activation of the ERK cascade [36] and promotes cell death. Since interfering with the MEKK1 pathway does not totally block apoptosis induced by cisplatin, it is reasonable to suggest that other parallel pathways such as ASK-1 [17] would be induced by cisplatin. In other situations, there may be other intracellular pathways regulated by JNK activation. For example, it has been described that in some systems, activation of JNK pathways may be a part of a DNA repair and cell survival mechanism [53].

Since the main intracellular target described for JNK is the

c-Jun protein, our results suggest that modulating the activity of this protein would have some effect in cisplatin-mediated apoptosis. Treatment of cells with inhibitors of gene expression, such as actinomicin D, has a protective effect in cells treated with cisplatin, suggesting that de novo gene expression is necessary for triggering the apoptotic process. In contrast with these results, Fas-induced apoptosis does not require new gene expression or protein synthesis and in this system, although JNK is activated, it is not necessary for apoptosis [32]. The results obtained using a dominant negative construction for c-jun that lacks the transactivation domain indicate that transcriptional activation of this protein contributes to cisplatin-induced apoptosis. In agreement with these results, expression of a c-jun gene lacking the transactivation domain is also able to protect sympatetic neurons against programmed cell death induced by NGF withdrawal [50]. We observed that AP-1 complexes that contained mainly c-Jun and ATF-2 proteins were induced after cisplatin treatment and remained high up to 6 h after treatment. These results are in agreement with the cisplatin-induced activation of both JNK and p38 with similar kinetics. On the other hand, AP-1 complexes containing c-Fos and c-jun increased with transient kinetics suggesting that AP-1 complexes containing c-Jun and ATF-2 could be involved in inducing the transcription of death genes. In agreement with these results, an increase in AP-1 complexes has also been reported to correlate with apoptosis in several physiological processes, such as involution of the mouse mammary gland and the rat prostate in vivo upon hormone depletion [54,55] or in response to DNA damaging agents [56–58].

Our results may have important implications in cancer chemotherapy with cisplatin. In addition to its toxic side effects, a major limitation in cancer chemotherapy is drug resistance. Our results suggest that deletions of genes encoding some of the proteins involved in transducing the death signal throughout the JNK cascade would result in increasing resistance to cisplatin, as occurs in the jun−/−-derived cells. Supporting this hypothesis, it has recently been reported that in knock out animals for JNK3, excitotoxicity fails to induce apoptosis in the hypocampus [59]. In a different study [60], it has been found that tumoral cell lines derived from pancreatic, lung, breast and colon carcinomas showed deletions in the encoding regions of the MKK4/SEK1 gene. These findings indicate that MKK4 may function as a suppressor of tumorigenesis by triggering apoptosis in response to stress and its mutations could contribute to drug resistance.

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