Inhibition of c-Jun-N-terminal-Kinase Sensitizes Tumor Cells to CD95-Induced Apoptosis and Induces G2/M Cell Cycle Arrest


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Abstract

Loss of susceptibility to apoptosis signals is a crucial step in carcinogenesis. Therefore, sensitization of tumor cells to apoptosis is a promising therapeutic strategy. c-Jun-N-terminal-kinases (JNK) have been implicated in stress-induced apoptosis, but may also contribute to survival signaling. Here we show that CD95-induced apoptosis is augmented by the JNK inhibitor SP600125 and small interfering RNA directed against JNK1/2. SP600125 potently inhibited methyl methane sulfonate–induced phosphorylation of c-Jun, but had minimal effect on apoptosis alone. In contrast, it strongly enhanced CD95-mediated apoptosis in six of eight tumor cell lines and led to a G2/M phase arrest in all cell lines. SP600125 enhanced cleavage of caspase 3 and caspase 8, the most upstream caspase in the CD95 pathway. JNK inhibition up-regulates p53 and its target genes p21Cip1/Waf1 and CD95. However, although HCT116 p53−/− cells and p21−/+ cells were less sensitive to CD95 stimulation than their p53+/+ and p21−/− counterparts, p53 and p21 were not involved in the JNK-mediated effect. JunD, which was described to be protective in tumor necrosis factor–induced apoptosis, was not regulated by JNK inhibition on the protein level. When transcription was blocked by actinomycin D, JNK inhibition still enhanced apoptosis to a comparable extent. We conclude that JNK inhibition has antitumor activity by inducing growth arrest and enhancing CD95-mediated apoptosis by a transcription-independent mechanism. (Cancer Res 2005; 65(15): 6780-8)

Introduction

CD95/Fas-induced apoptosis is an important mechanism for physiologic cell homeostasis and pathophysiology. CD95-mediated apoptosis plays a major role in fulminant liver failure, hepatitis, and cholestatic liver disease (1). In contrast, impairment of CD95-mediated apoptosis may contribute to carcinogenesis of different tumors (2). CD95 expression is dependent on the function of the tumor suppressor protein p53 (3), which is impaired in ~50% of all human tumors. In particular, in hepatocellular carcinomas, loss of p53 and CD95 expression or expression of inhibitory molecules of the CD95 pathway is associated with dedifferentiation, larger tumor size, and poor prognosis and is a predictor of tumor recurrence (4, 5). Thus, activation or silencing of the CD95 pathway contributes to pathophysiologic processes. Specific inhibition or activation of the CD95 pathway are hence novel and important therapeutic goals.

CD95 is a member of the tumor necrosis factor (TNF) receptor superfamily. Activation of CD95 by its specific ligand, CD95L/FasL, induces apoptosis in most cell types, as CD95 is ubiquitously expressed in the human body (6). After activation of CD95, the adapter protein, Fas-associating protein with death domain, binds to the death domain of CD95 and attracts procaspase 8 via its death effector domain to the receptor complex, the death inducing signaling complex (DISC). Upon DISC formation, caspase 8 is autolytically cleaved and activated and, in turn, cleaves downstream caspases such as effector caspase 3, leading to cleavage of cellular proteins and DNA and to subsequent programmed cell death (7).

Via cleavage of Bid, caspase 8 is also able to stimulate mitochondria to release apoptogenic molecules such as apoptosis activating factor 1, cytochrome c, and ATP, leading to activation of effector caspases. Whether active caspase 8 preferentially activates caspase 3 directly or the mitochondrial pathway depends on the cell type (type I versus type II cells; ref. 8).

The mitogen-activated protein kinase (MAPK) cascade is one of the central cellular signaling pathways and the MAPK kinase (MEKK) 1-MAPK kinase 4/MAPK kinase 7-c-Jun-N-terminal-kinase (JNK) 1/2-c-Jun axis seems to be important in the regulation of apoptosis. c-Jun knockout in mice (9) is embryonically lethal due to increased apoptosis in the liver, indicating a prominent role of c-Jun in liver cell survival. Recently, we showed that the MEKK/JNK pathway operates upstream of the CD95 pathway to mediate chemotherapeutic drug–induced apoptosis in liver cells via a c-Jun responsive element (10). In hepatocellular carcinoma, MAPKs are often constitutively activated (11–14) and c-Jun promotes liver carcinogenesis (15). Therefore, we reasoned that death receptor–induced apoptosis may be suppressed via interaction with MAPK/JNK family members.

In this study, we show that inhibition of c-Jun phosphorylation via inhibition of JNK1/JNK2 activity by SP600125 or small interfering RNA (siRNA) strongly enhances CD95-mediated apoptosis, whereas JNK inhibition alone has little effect on cell survival. This effect is cell line specific because it was readily observable in HepG2 hepatoma cells, Jurkat lymphoma cells, as well as HT29 and HCT116 colon carcinoma cells, but not in Huh7 or Hep3B hepatoma cells. In addition, p53 and its transcriptional targets p21 and CD95 were up-regulated upon treatment with SP600125. However, the increase in apoptotic activity after CD95 cross-linking was not dependent on p53 as determined by HCT116 p53-positive and p53-negative cells. The effect was due to an increase in
activities of caspase 8 and caspase 3, indicating an interaction between the CD95 and JNK pathways at the level of the CD95 DISC. Moreover, inhibition of JNK led to growth inhibition via G2/M arrest. Our data suggest the inhibition of JNK as a potential tumor therapy, especially for tumors which have down-regulated the CD95 death receptor system during tumorigenesis such as hepatocellular carcinoma (5).

Materials and Methods

Cell lines. HepG2, Chang liver, and Jurkat cells were grown in RPMI medium (PAA Laboratories, Cölbe, Germany); HCTL11 p53+/− (clone p40-1), p53−/− (clone 379.2), p21+/−, and p21−/− cells were cultured in McCoy’s 5A medium (Gibco BRL, Eggenstein, Germany); and HT29 cells were cultured in DMEM (Gibco BRL). All media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco BRL).

Reagents. SP600125 (Tocris, Cologne, Germany) was dissolved in DMSO as a 20 mmol/L stock solution and stored at −20 °C. To avoid precipitation of SP600125, prewarmed media were used and DMSO was added to a final concentration of 0.2%.

Measurement of apoptosis and cell cycle analysis. Apoptosis and cell cycle distribution were analyzed using flow cytometry according to Nicoletti et al. (16). Cells were scraped with a rubber policeman, washed with PBS, and incubated in staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide overnight. Sub-G1 events and cell cycle distribution were measured in a fluorescence-activated cell sorter, FACScalibur (Becton Dickinson), using Cell Quest as software. Specific rate of apoptosis was calculated as (rateprobe − ratetreatment) / (100% − ratetreatment).

Western blot analysis. Western blot analysis was carried out as described previously (17). Briefly, proteins were separated by SDSPAGE or by Bis/Tris precast gels (Invitrogen, Karlsruhe, Germany) and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour in TBS solution containing 0.1% Tween 20 (TBST) and 5% milk powder. Membranes were then incubated with primary antibodies in TBST at 4 °C overnight, followed by the appropriate horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. Westerns were developed using the ECL system (Amersham, Braunschweig, Germany) or Western blots of chromogenic immunodetection kit (Invitrogen). The following antibodies were used: caspase 3 (H-277; Santa Cruz, Heidelberg, Germany), p53 (DO-1; Santa Cruz), C4 (BD Transduction, Lexington, KY), phospho-c-Jun (Ser63) II (Cell Signalling), c-Jun (BD Transduction), CD95 (C-20; Santa Cruz), JunD (329; Santa Cruz), and actin (clone C8; ICN Biomedicals, Eschwege, Germany). The caspase 8 and c-FLIP antibodies were a kind gift from Peter Krammer and have been described previously (8, 18). Densitometric analysis was carried out using TINA software (Raytest GmbH, Straubenhardt, Germany).

Reverse transcription-PCR. RNA was prepared from 2.5 × 10⁴ cells grown in 100 mm dishes for each probe, using the RNaseasy kit (Qiagen GmbH, Hilden, Germany) following the instructions of the manufacturer. Reverse transcription-PCR (RT-PCR) was done as described before (10). Briefly, 1 μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Eggenstein, Germany) with oligo(dT)15 primers (Roche GmbH, Penzberg, Germany) in a 20 μL reaction tube containing 10 mmol/L DTT and 500 μmol/L deoxynucleotide triphosphates. One-microliter aliquots were amplified in a DNA thermocycler (MJ Research, Watertown, MA) with 0.5 unit of Taq DNA polymerase (Roche) in a 50 μL reaction. Thirty reaction cycles were done. Each cycle consisted of a denaturation step (94 °C for 30 seconds), an annealing step (56 °C for 30 seconds), and an elongation step (72 °C for 10 minutes). PCR products were analyzed in agarose gels. Primers were purchased from MWG Biotech GmbH (Ebersberg, Germany). Primer sequences were as follows: CD95 sense 5′-CACTCCTGCAACCTCTCCTCCC-3′ and antisense 5′-AGAGTTGTGTCACAAGGCTG-3′, yielding a product of 400 bp; human glyceraldehyde-3-phosphate dehydrogenase sense 5′-ACCACAG-TCCATGCTCATAC-3′ and antisense 5′-TCCACACACTCTTGCTGTA-3′, yielding a product of 450 bp.

RNA interference. The following sequence was used for the design of siRNA specific for both JNK1 and JNK2: sense 5′-TGAAAGAATGCC-TACCTT-3′ and antisense 5′-AAGGTAGGACATCTTTCCTCA-3′. Controls were done with sequences against green fluorescent protein: sense 5′ AAG GT CAG TCC AGG AGC GCA 3′ and antisense 5′ AAT CGG CTC CTG GAC GTA GCC 3′. siRNAs were synthesized using the Silencer siRNA Construction Kit (Ambion, Austin, TX) following the instructions of the manufacturer. For transient transfection, Oligofectamine (Invitrogen) was used according to the protocol of the manufacturer. Cells (1 × 10⁶) were seeded in 12 wells 24 hours before transfection in RPMI without antibiotics. Transfection was carried out in OptiMEM (Gibco) without FBS, which was added after 5 hours. Two- and a half days later, cells were treated with anti–APO-1 antibody as indicated for 16 hours. Cells were pooled and 50% were used for fluorescence-activated cell sorting analysis (FACS) and Western blot, respectively.

Results

SP600125 augments CD95-induced apoptosis. JNK has been shown to have positive, negative, or no influence on apoptosis under different experimental conditions (19, 20). To study the influence of JNK on CD95-mediated apoptosis, we used HepG2 cells as a model system. The cells were treated with the agonistic anti-CD95 antibody anti–APO-1 or the JNK inhibitor SP600125. Here and in the following experiments, protein A at 0.01 times the anti–APO-1 concentration was used to facilitate cross-linking unless indicated otherwise. Apoptosis was quantified by flow cytometry and measurement of sub-G1 events according to Nicoletti et al. (16). Treatment of cells with 0.5 μg/mL anti–APO-1 for 16 hours induced up to 40% apoptosis, in agreement with our previous observations (Fig. 1A; ref. 10). Treatment with 20 μmol/L SP600125 alone had little effect on apoptosis, as had treatment with DMSO. However, combination of 20 μmol/L SP600125 with 0.5 μg/mL anti–APO-1 enhanced apoptosis, indicating that JNK acts to suppress CD95-induced apoptosis (Fig. 1A).

Kinetic analysis with anti–APO-1 and SP600125 showed that the effect is specific and can be detected after 4 hours with a difference of 20% after 16 hours (Fig. 1B, top). In another set of experiments, prolonged treatment showed that this effect was maintained for at least 36 hours, indicating that the substances are still active and that the effect is not due to a mere delay of CD95 signaling (Fig. 1B, bottom). Apoptosis rates for DMSO, SP600126, anti-APO, and SP600126 plus anti-APO were 4.3%, 10.2%, 31.3%, and 52.6% after 24 hours, respectively, and 2.9%, 13.9%, 36.3%, and 60.5% after 36 hours.

SP600125 induces G2-M arrest in HepG2 cells. Activation of JNK and phosphorylation of c-Jun has been shown to be closely linked to cell cycle regulation. In particular, inhibition of c-Jun by transfection with a dominant negative form of the protein induced a G1 arrest in different cell lines (21). Therefore, we investigated the effect of SP600125 on cell cycle progression. HepG2 cells were treated with 20 μmol/L SP600125. SP600125 induced a reversible G2-M arrest in HepG2 cells (Fig. 1C and data not shown). Kinetic analysis of cell cycle progression revealed an increasing G2-M fraction after a 16-hour observation period, being apparent after 4 hours of incubation with SP600125 (Fig. 1D).

c-Jun phosphorylation is inhibited by SP600125 treatment and c-Jun-N-terminal-kinase–directed small interfering RNA sensitizes cells to CD95-mediated apoptosis. To show that SP600125 is able to inhibit JNK in our system, Western blot analysis of HepG2 cells was done using phospho-specific
antibodies against c-Jun. Treatment with SP600125 had no detectable effect on the phosphorylation status of c-Jun due to low baseline phosphorylation. When cells were stimulated by the alkylating agent methyl methane sulfonate (22), c-Jun phosphorylation was strongly enhanced in a dose-dependent manner (Fig. 2A), and a potent inhibitory effect of 20 μmol/L SP600125 was readily detectable in our system. Cotreatment with SP600125 for 2 hours almost completely abolished c-Jun phosphorylation. Treatment with SP600125 and addition of methyl methane sulfonate after 14 hours showed that the substance is stable and active after overnight treatment. Total protein levels of c-Jun were also elevated after treatment with methyl methane sulfonate relative to actin protein levels, confirming a positive feedback loop of phospho-c-Jun on its own promoter (Fig. 2A; ref. 23). In conclusion, SP600125 is a potent inhibitor of c-Jun phosphorylation in our system.

To inhibit JNK by an additional approach, we used a newly designed siRNA directed against a common sequence of JNK1 and JNK2. Figure 2B shows that transient transfection with this siRNA, but not with green fluorescent protein control siRNA, led to efficient down-regulation of both JNK1 and JNK2, and that this down-regulation was not influenced by subsequent treatment with anti-APO (Fig. 2B, left). The values of densitometric analysis are indicated below the figures. Moreover, down-regulation of JNK1/2 by siRNA efficiently inhibited methyl methane sulfonate–induced phosphorylation of c-Jun (Fig. 2B, right). As we had observed after application of SP600125, JNK inhibition by siRNA alone did not induce significant rates of apoptosis after 16 hours but clearly increased the sensitivity to CD95 stimulation (Fig. 2C).

SP600125 exerts a cell type–independent but genotype-dependent effect. We investigated whether the apoptosis-enhancing effect of SP600125 is specific for liver cells. To this end, we treated HepG2 cells, Chang hepatoblastoma cells, HT29 colon carcinoma cells, and the T-lymphoblastoid cell line Jurkat with anti–APO-1 antibodies, either alone or in combination with SP600125. CD95-mediated apoptosis was enhanced by SP600125 in all of these cell types (Fig. 3A). Apoptosis rates by anti-CD95 treatment were 46.8%, 26.5%, 14.1%, and 42.5% in HepG2, Chang liver, HT29, and Jurkat cells, respectively. Addition of SP600125 to cell cultures led to increases of 68.7%, 54.8%, 32.4%, and 74.9%, respectively. Thus, the effect of SP600125 is not limited to liver cells, but is also present in cell lines originating from colon and lymphoid tissue. However, when Huh7 or Hep3B liver carcinoma cells were treated under the same conditions, no effect of either anti CD95, SP600125, or the combination was observed. In these two cell types, apoptosis cannot be efficiently induced by stimulation of CD95 (24) and SP600125 did not have an effect on cell death (Fig. 3A).

In contrast, the growth arrest induced by SP600125 was similar in HepG2, Jurkat, Huh7, and Hep3B cells (Figs. 2 and 3B). Cell cycle arrest in Chang liver cells was more pronounced, reaching almost 60% G2/M fraction, whereas G2/M arrest was only marginally induced in HT29 colon carcinoma cells (Fig. 3B).
Inhibition of c-Jun-N-terminal-kinase 1/2 facilitates CD95-mediated activation of caspase 8 and caspase 3. To determine the level of interaction between the CD95 and MAPK pathways, we investigated caspase activation downstream of CD95. Activation of the effector caspase 3 was increased after treatment with SP600125 and CD95 activation for 16 hours compared with CD95 activation alone, as judged by appearance of cleavage products, in particular p11 (Fig. 4A). Treatment with SP600125 alone or vehicle did not induce caspase-3 cleavage. Next, we looked at activation of the initiator caspase 8. Similar to caspase 3, cotreatment with anti–APO-1 and SP600125 resulted in stronger activation of the caspase, as the p18 fragment was detectable in higher amounts, whereas the amount of procaspase (p55) was decreased. Like caspase 3, caspase 8 was not significantly activated after treatment with SP600125 or vehicle alone (Fig. 4A).

Thus, inhibition of JNK1/2 leads to changes at the level of CD95 DISC or procaspase 8.

Role of p53 and its target genes p21Cip1/Waf1 and CD95 in sensitization to apoptosis and G2-M arrest by c-Jun-N-terminal-kinase inhibition. A possible explanation for the enhancement of CD95-mediated apoptosis was provided by the recent finding that c-Jun represses p53 (15), which is a known activator of CD95 transcription (3). To determine the role of p53 in the crosstalk between the MAPK cascade and the CD95 pathway, we investigated the influence of JNK inhibition on p53 levels. Treatment with SP600125 led to induction of p53 protein in HepG2 cells (Fig. 4B). A dose-dependent induction of p53 was also observed in HCT116 p53+/− cells (Fig. 4C). Correspondingly, JNK inhibition by SP600125 led to induction of the p53 target genes p21Cip1/Waf1 (Fig. 4B and C). However, CD95 stimulation was not observed in the p53−/− cells, indicating a link between JNK and CD95 via p53.

Apoptosis was induced in both HCT116 p53+/+ and p53−/− cells by CD95 stimulation and apoptosis rates were increased by concomitant JNK inhibition (Fig. 5A). General rates of apoptosis were lower in HCT116 p53−/− cells; however, the effect of JNK inhibition was comparable between p53-positive and p53-negative HCT116 cells (Fig. 5A). Furthermore, the cell cycle inhibitor p21Cip1/Waf1, which also modulates CD95-mediated apoptosis (25, 26), strongly inhibited apoptosis, but had no effect on the sensitizing effect of JNK inhibition (Fig. 5B).

Figure 2. JNK inhibition inhibits methyl methane sulfonate–induced phosphorylation of c-Jun and siRNA against JNK sensitizes cells to CD95-mediated apoptosis. A, Western blot analysis of phospho-c-Jun, c-Jun, and actin (loading control) in HepG2 cells. Cells (3.5 × 10⁶) were seeded in 10 cm dishes. After 24 hours, cells were treated with 20 μmol/L SP600125 and/or methyl methane sulfonate as indicated. Asterisk, methyl methane sulfonate was added 10 minutes after cells were incubated with SP600125 (lanes 3 and 7). Lane 5, methyl methane sulfonate was added 14 hours after the addition of SP600125. Thirty micrograms of total protein per lane were separated by SDS-PAGE. B and C, siRNA (10 nmol/L) directed against JNK1/2 or green fluorescent protein was transiently transfected into HepG2 cells. Left, anti–APO-1 was added at 0.5 μg/mL for 16 hours where indicated and JNK expression was detected by Western blot. Numbers below the graph indicate band intensities relative to the respective actin band and relative to mock-transfected cells. Values of the top line represent the upper band of JNK; values of the bottom line represent the lower JNK band. Right, Western blots of c-Jun and phospho-c-Jun expression with and without treatment with 1 mmol/L methyl methane sulfonate for 2 hours. Values represent band intensities; top values refer to phospho-c-Jun; bottom values refer to c-Jun levels relative to the respective actin band and relative to mock-transfected cells. C, 50% of the cells used in (B, left) were used for FACS analysis. Columns, mean apoptosis rates (sub-G1 fractions) from three independent experiments; bars, SD.
conclusion, JNK inhibition has an apoptosis-sensitizing effect on the CD95 pathway upstream of caspase 8 and independent of p53, p21, and CD95 itself.

Likewise, the G2/M arrest on SP600125 treatment was similar between HCT116 p53+/+ and p53−/− cells (Fig. 5A) and between HCT116 p21+/+ and p21−/− cells (Fig. 5B), indicating a p53- and p21-independent mechanism.

c-Jun-N-terminal-kinase inhibition enhances CD95-mediated apoptosis independently of JunD and transcription. Recently, it has been demonstrated that JunD acts as a survival factor in the JNK signal transduction pathway. Thus, JNK increases JunD expression leading to enhanced apoptosis resistance (27). This function of JunD is consistent with the established role of this transcription factor as an inhibitor of TNF-stimulated apoptosis (28). As shown in Fig. 6A, treatment of HepG2 cells with SP600125 has no influence on cellular JunD levels. Therefore, the JNK-mediated survival signal does not involve the up-regulation of JunD in our system.

To determine whether the transcription of other genes is involved in the effect of JNK on CD95 signaling, we blocked mRNA transcription with actinomycin D. Figure 6C shows that actinomycin D did not abrogate the sensitizing effect of SP600125. Thus, direct protein-protein interactions independent of transcription are likely to account for the sensitization.
Discussion

We show here that inhibition of JNK by SP600125, the first selective JNK inhibitor, and by siRNA directed against JNK1 and JNK2 enhances specific rates of apoptosis induced by the CD95 death receptor 1.5- to 2-fold after 16 hours in which no further increase was achieved with higher concentrations of anti–APO-1 antibody (Figs. 1A and B, 2C, 3A, 5A and B, and 6 and data not shown). In addition, cell cycle progression is halted at the G2/M phase of the cell cycle.

The JNK pathway has been shown to be closely linked to apoptosis (29). Recently, we showed that the MEKK-1/JNK kinase/JNK/c-Jun axis operates upstream of the CD95 pathway to mediate chemotherapeutic drug–induced apoptosis (10), acting proapoptotically. Conversely, deletion of single components of the JNK pathway also results in apoptosis and c-Jun knockout mice as well as MAPK kinase 4 knockout mice die of increased apoptosis in the liver (30). Because the exact role of JNK in apoptosis seems to depend on the cell type and the apoptotic stimulus, more thorough investigation is needed.

SP600125 was described as an inhibitor of the JNK pathway for the treatment of autoimmune, inflammatory, and neurodegenerative diseases (31). SP600125 was shown to be selective for JNK1/JNK2 and, to a somewhat lesser extent, for JNK3, which is expressed in certain neuronal and testicular cells (31). In addition to SP600125, we used an siRNA directed against both JNK1 and JNK2, which showed the same effect as SP600125 (Fig. 2).

The role of JNK in TNF-α–induced apoptosis has been extensively studied. Although early contradictory reports exist (32, 33), it has been repeatedly shown to have a proapoptotic role in TNF-treated cells in which the NF-κB pathway was blocked (34–36). A proapoptotic role of JNK has also been
described in apoptosis induced by different chemotherapeutic agents, such as vinblastine, doxorubicin, and etoposide (37, 38). Furthermore, phenethyl isothiocyanate–mediated apoptosis was inhibited by SP600125 in colon adenocarcinoma cells (39); troglitazone-induced apoptosis was inhibited in HepG2 cells (40); and 2-methoxyestradiol–induced apoptosis was inhibited by SP600125 in multiple myeloma cells (41). In contrast, Vivo et al. (42) showed that JNK inhibits TRAIL-induced apoptosis.

Less clear is the role of JNK in CD95-mediated apoptosis. A link between JNK and the CD95 pathway is provided by the finding that c-Jun exerts activator protein 1–mediated suppression of the CD95 promoter (15, 43). Furthermore, c-Jun was described to suppress the transcription of p53, which is a known regulator of CD95 expression (44). In agreement with these observations, we found up-regulation of p53 and CD95 upon treatment with SP600125 (Fig. 4B).

Although the apoptosis-enhancing effect of the JNK inhibitor was not present in p53-mutated Huh7 and p53-deficient Hep3B cell lines (Fig. 3A) and apoptosis rates in p53 null cells were generally lower, we observed no significant difference as to the enhancing effect of SP600125 between p53-positive and p53-negative cell lines (Fig. 5A). Therefore, we conclude that neither p53 nor CD95 expression levels contribute to the derepression of CD95-induced apoptosis. Similarly, the p53 target gene...
protein-protein interactions between CD95 and JNK signaling components are likely to account for the sensitizing effect. JunD was suggested as a mediator of JNK survival signaling in TNF-induced cell death (27), as increased JunD expression downstream of JNK signaling leads to increased apoptosis resistance. However, in our system JunD is not regulated on JNK inhibition and therefore does not account for the sensitizing effect (Fig. 6A).

Further studies are required to elucidate the mechanism of apoptosis sensitization.

It has been shown in breast cancer cells that JNK inhibition leads to endoreduplication (DNA content > 4N; ref. 38). In agreement with this data, endoreduplication was detectable in HepG2 cells (Fig. 1A) and was much more pronounced in HCT116 cells (data not shown). In addition, we show that inhibition of JNK signaling leads to cell cycle arrest at the G2/M boundary. Furthermore, p21Cip1/Waf1 is up-regulated at the protein level. This observation is in line with earlier data (21). However, whereas Potapova et al. described that growth arrest is maximized in p53 null cells, growth inhibition in our experimental system is independent of p53, as p53-negative cells show comparable levels of apoptosis and cell cycle arrest. One possible explanation is the selective inhibition of JNK2 (21), whereas SP600125 inhibits JNK1 and JNK2 to a similar extent.

Although target genes of p53, such as GADD45, 14-3-3ζ, and p21Cip1/Waf1, are able to cause cdc2 inhibition and subsequent G2 arrest (47), G2/M arrest was also induced in p53-negative cells (HCT116 p53−/−, Hep3B, and Huh7), leading to the conclusion that p53 is not crucial for the observed effect as was the case with the effect on apoptosis.

In conclusion, we show that CD95-induced apoptosis is augmented by inhibition of JNK1 and JNK2. Moreover, SP600125 halts cell cycle progression in tumor cells at the G2/M boundary. Thus, inhibition of JNK is an interesting goal for tumor therapy. In particular, tumors which down-regulate CD95 during tumorigenesis (48) or have a CD95 system with a reduced activity due to the inhibition of expression of inhibitory molecules are promising candidates for this approach.

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