p73-Dependent Apoptosis through Death Receptor: Impairment by Human Cytomegalovirus Infection

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Abstract

The discovery of p73, a p53-related protein with various isotypes resulting from different promoter usage or splicing events, provided new insights into regulation of neurogenesis and tumorigenesis. Among p73 isoforms described thus far, TA-truncated molecules (ΔN) appeared as key proteins according to their antagonistic activity against transcription factor activity of p53 family members. We previously showed that infection by human cytomegalovirus (HCMV) induced drug resistance and altered p53- and p73-dependent apoptosis of infected cells through accumulation of ΔN-p73α. In accordance with the ability of p53 to induce apoptosis through death receptors, we asked whether p73 activation could compensate for p53 deficiency. We showed that p73 transcriptional activity sensitized cells to apoptosis through death receptors in a caspase-dependent pathway. Expression of the death-inducing signaling complex (DISC) proteins was unchanged, whereas p73 activation through either cisplatin treatment or ectopic overexpression induced up-regulation of Fas transcription and expression at cell surface. According to its ability to flood cells with ΔN-p73α, HCMV inhibited p73-dependent Fas-mediated apoptosis, gaining an additional trick to favor its survival in the host cell. Owing to the involvement of p53- and p73-dependent death receptor signaling in development of the central nervous system, immune surveillance of neural cells, and sensitivity of tumors to drugs, our previous and present data prompt us to consider stabilization of ΔN-p73α by HCMV as a possible mechanism in impairment of embryogenesis and in tumorigenesis. (Cancer Res 2005; 65(7): 2787-94)

Introduction

Apoptosis plays a critical role in important biological processes such as development and adult tissues homeostasis for the elimination of superfluous, virally infected, transformed, and damaged cells. Impaired control of apoptosis may be a significant factor in the etiology of a number of diseases such as neurodegenerative disorders, cancer, autoimmunity, and viral infections. Since its discovery, the tumor suppressor p53 has been in the forefront according to its role as a sensor of DNA damage and its ability to direct transcription of genes involved in DNA repair, apoptosis, and recognition of malignant cells by T lymphocytes (1, 2). Accordingly, inactivation of p53 is the most common defect in cancer cells. Its privileged position has been softened recently by the discovery of the p53-related genes, *TP63* and *TP73*, owing to their distinctive abilities to control genes involved in stem cell identity, neurogenesis, natural immunity, and to act as repressors of p53 activity (3–6). Both genes encode various isotypes resulting from using separate promoters and differential splicing. Two classes of products have been identified: those containing a transactivating domain exhibiting p53-like functions and those truncated at the NH_2-terminal end, ΔN-p63 and ΔN-p73α, with antagonistic effects on p53 and p73 functions (6). Thus, ΔN isoforms could be considered as key regulators in many normal and pathologic processes, including embryogenesis, cell differentiation, tumorigenesis, and immune surveillance. Interestingly, cross-talks may exist between p53- and death receptor–mediated apoptosis through transcription-dependent and -independent pathways (7), and in various occasions, p63 and p73 can exert p53-like activities and act as inducers of apoptosis by transactivating p53 target genes (8, 9). It has been suggested that tumor necrosis factor receptor (TNFR)–related proteins such as Fas and DR5 may participate in DNA damage–induced cell death through p53-dependent regulation and that the induction of p53 expression and stimulation of the Fas pathway represent a major mechanism by which cytotoxic drugs induce apoptosis (10).

Whether p73 could compensate for p53 deficiency and whether the effects of ΔN isoforms regulatory activity could be broadened to all the p53- and p73-dependent pathways, including death receptors signaling, remain opened questions. We provided the first evidence that viruses could take advantage of ΔN inhibitory activity. We showed that human cytomegalovirus (HCMV), a member of the betaherpes virus family, induced drug resistance and altered p53- and p73-dependent apoptosis in p73-positive infected neuroblastoma and astrocytoma cells through accumulation of ΔN-p73α (11). Our data provided molecular bases for HCMV-associated abnormal embryonic development and neurologic defects in newborns and for resistance of HCMV-infected neuroblastoma to drugs. HCMV can be considered as a spearhead in exploiting coexistence with the host to develop numerous evasion mechanisms, including blockade of apoptosis and of recognition of infected cells by T lymphocytes (12–14). According to the ability of HCMV to induce stabilization of ΔN-p73α, we made the assumption that in p73-positive cells, infection may interfere with p53-dependent death receptors signaling.

In the present report, we first showed that in p73-positive cells, p73 transcriptional activity can induce sensitivity to apoptosis through death receptors involving activation of initiator and executioner caspases. We showed that expression levels of the death-inducing signaling complex (DISC) proteins FADD, FLICE, and c-FLIP were not modified, but that in contrast, Fas expression was up-regulated following either p73 activation by cisplatin

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or ectopic overexpression. Furthermore, according to its ΔN-inducing effect, infection of p73-positive targets by HCMV inhibited up-regulation of Fas expression under cisplatin treatment and thus participated in blocking sensitization to death. Our data suggest that p73 protein could compensate for p53 deficiency in its ability to sensitize targets to death ligands and show how HCMV gained an additional trick to elude host surveillance and new clues on how the virus may persist and contribute to favor tumor development and to impair sensitivity to drugs.

Materials and Methods

Cell cultures. SK-N-AS neuroblastoma and U373MG astrocytoma cell lines were from American Type Culture Collection (Molsheim, France). SK-N-AS were maintained in DMEM and U373MG in RPMI (Life Technologies, Cergy Pontoise, France) both containing 10% FCS (Life Technologies) and supplemented with 10 μg/mL Oflocet anti-*Mycoplasma* (Roussel, Paris, France) and sodium pyruvate, unless otherwise stated. Transcript sequences and protein expression of p73 and p53 have been described in ref. (3): SK-N-AS express p53 but undetectable levels of p73 (p53+, p73−) and U373MG express wild-type p73 and mutant p53 (p73+, p53−).

Viruses. The laboratory strain of HCMV AD169 (American Type Culture Collection) was propagated in human foreskin fibroblasts (HFF) in DMEM supplemented with 5% FCS. Virus was collected when cytopathic effects were >90%. Supernatants were clarified of cell debris by centrifugation at 1,500 × *g* for 10 minutes at 4°C and were stored at −70°C until use. Virus titers were determined by plaque-forming unit titration in HFF according to standard procedures. A multiplicity of infection (MOI) of 1 was used for all the experiments.

Type 5 adenovirus either empty (Ad-Mock) or recombinant for p73α (Ad-p73α), ΔN-p73α (Ad-ΔN-p73α), and p73-M293 (Ad-p73-M293) were obtained as described previously (11). Adenoviruses were used at MOI of 50 in serum-free medium. Virus was removed after 4 hours of incubation and cells were incubated in normal medium for further 20 hours.

Small interfering RNA design. We used the plasmid-based system psiRNA from Invivogen (San Diego, CA), which expresses a zeocin-resistant marker, to generate stable small interfering RNA (siRNA) into U373MG cells. Oligonucleotides encoding two scramble sequences (universal control from Invivogen) or two complementary sequences homologous to a segment of ΔN-p73 were synthesized (respectively 5′-CCCATTCAgCCAgTTgACAgAACTTCAAgAgAgTTCTgTCAACTggCTgAATTT-3′, 5′-AAAAAATTCAgCCAgTTgACAgAACTCTCTTgAAgTTCTgTCAACTggCTgAAT-3′ and 5′-CCCCAgCAgCTgTACAgAATTATCTCAAgAggATAATTCTgTACAgCTgCTgTT-3′ and 5′-CAAAAACAgCAgCTgTACAgAATTATCCTCTTgAgATAATTCTgTACAgCTgCTg-3′).

All siRNA sequences were subjected to blast search to confirm the absence of homology to any additional known coding sequence in the human genome. After hybridization, oligonucleotides were cloned into the *Bbs*I site of psiRNA, and the plasmids were sequenced and amplified. U373MG cells were transfected using Lyovec reagent (Invivogen) according to manufacturer instructions. Twenty-four hours after transfection, the selection agent zeocin (Invitrogen SARL, Cergy Pontoise, France) was added in the culture medium at 150 μg/mL during 15 days.

Stably resistant U373MG cells were analyzed for their sensitivity to apoptosis using propidium iodide and FITC-conjugated Annexin V double labeling, and the ΔN-p73α level was checked using Western blot as described further.

Semiquantitative reverse transcription-PCR experiments. Total RNA was extracted from mock- and HCMV-infected cells using the TRIzol LS reagent (Invitrogen). cDNAs were obtained by reverse transcription using the Superscript preamplification system kit (Invitrogen) with 3 μg of total RNA. PCR amplification was carried out using the following primer pairs: Fas (sense 5′-CTgCATgTTTTCTgTACTTCC-3′ and antisense 5′-ggATgAACCAgACTgCgTg-3′); IE1 (sense 5′-gTgTgATgCTggCCAAgCgg-3′ and antisense 5′-ggTCAgCCTTgCTTCTAgTC-3′); UL36 (sense 5′-CgACTACCAgCggCCCATgg-3′ and antisense 5′-gAggAAgCCCATgTAggTCT-3′), UL37 (sense 5′-AggTAgCgTgggCCTgCTgg-3′ and antisense 5′-gAACCggTgAgAggCgTCCC-3′); β-Actin (sense 5′-gAAgCATTTgCggTggACgAT-3′ and antisense 5′-TCCTgTggCATCCACgAAACT-3′) with 33 cycles (95°C at 30 seconds, 60°C at 1 minute, and 72°C at 1 minute).

Cell death assays. U373MG or SK-N-AS cells were cultured in 6-well culture. Cells were treated or not for 2 hours with cisplatin (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 25 μmol/L final concentration and washed and incubated with either agonist anti-Fas IgM (200 ng/mL, clone 7C11, Immunotech), or rhsTNF-α (200 ng/mL, R&D Systems, Lille, France) or rhsTRAIL plus Enhancer (50 ng/mL and 2 μg/mL, respectively; Alexis Co., Illkirch, France) for additional 24 hours. Alternatively, before treatment with cisplatin and apoptotic ligands, cells were infected with the HCMV laboratory strain AD169, or adenoviruses as mentioned or treated with either caspase inhibitors: Z-VAD-FMK, Z-IETD-FMK, or Z-LEHD-FMK (50, 20, and 20 nmol/L, respectively; all from Calbiochem, La Jolla, CA). The presence of apoptotic cells was detected by multiparameter flow cytometry (Coulter Epics) using propidium iodide and FITC-conjugated Annexin V (Coulter-Immunotech, Marseille, France) double labeling according to manufacturer's instructions. Cell death was also detected by Western blotting of poly (ADP-ribose) polymerase (PARP) cleavage as described below or by counting the trypan blue (Life Technologies) colored cells. The percentage of dead cells was calculated as follows: [(number of colored dead cells / number of total cells) × 100]. Activation of caspase 3 was assessed by using the caspase-3 apoAlert colorimetric assay kit (DAKO S.A., Trappes, France) or flow cytometry using a specific FITC-conjugated anti-active-caspase-3 antibody according to manufacturer's instructions (R&D Systems).

Western blotting. U373MG cells cultured in 6-well plates were infected and treated as indicated. Cells were detached with trypsin and washed with PBS. Cell lysates in lysis buffer (150 mmol/L NaCl, 1% NP40, 0.5% D.O.C, 0.1% SDS, 50 mmol/L Tris) were homogeneized with a pipette and centrifuged for 15 minutes at 10,000 rpm. Supernatants were collected and total proteins were quantified using a Bio-Rad protein assay kit (Bio-Rad, Marnes La Coquette, France). Thirty micrograms of proteins were boiled for 5 minutes in 5% β-mercaptoethanol reducing Laemli sample buffer, and samples were separated by SDS-PAGE in 10% denaturing polyacrylamide gels and transferred to nitrocellulose membrane (Hybond C, Amersham, Piscataway, NJ). Immunoblots were stained with Ponceau red to visualize total proteins contained in each slots and probed alternatively with the following antibodies: rabbit polyclonal anti-human p73α (15) used at 1:2,000 dilution; PARP mouse monoclonal antibody (mAb, clone C-2-10, 1:10,000 dilution; Clontech Laboratories, Palo Alto, CA), FADD mouse mAb (clone A66-2, 1:1,000 dilution; BD PharMingen, San Diego, CA), c-FLIP-S rabbit antibodies (H-202, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), c-FLIP-L rabbit antibodies (H-150, 1:500 dilution; Santa Cruz Biotechnology), FLICE mouse mAb (clone IC12, 1:500 dilution; Cell Signaling Technology, Beverly, MA), Fas Death domain mouse mAb (clone 3D5, 1:1000 dilution; Alexis Biochemicals), and β-actin mouse mAb (clone AC-15, 1:5000, Sigma). Blots were revealed using peroxidase-conjugated polyclonal anti-rabbit antibody (1:5,000 dilution, Transduction Laboratories, San Diego, CA) or peroxidase-conjugated polyclonal anti-mouse antibody (1:10,000 dilution, Sigma) and enhanced chemiluminescence detection kit (Amersham). Semiquantification of Western blotting was done using SigmaGel software.

Flow cytometry. Cells were cultured in 6-well plates and treated or infected as indicated. For Fas labeling, cells were detached with trypsin and washed with PBS-3% FCS. Anti-Fas-PE (clone DX2, mouse, DakoCytomation, Trappes, France) was applied directly onto pelleted cells. Cells were incubated for 45 minutes at 37°C. Labeling with an isotypic mouse IgG1 served as a control. Cells were washed with PBS-3% FCS and analyzed with a Coulter EPICS Elite cell sorter.

Results

Sensitization of cisplatin-treated cells to apoptosis through death receptors. Induction of DNA damage and apoptosis by cisplatin can involve either p53- or p73-dependent pathways (16). We previously showed that sensitivity of U373MG astrocytoma

cells (p73+, p53−) to apoptosis by cisplatin required p73 activity (11). It has been established that expression of death receptors can be induced by DNA damage and contribute to DNA damage-induced apoptosis in a p53-dependent manner (17, 18). Accordingly, we asked whether signaling through death receptors could be switched on following activation of p73. To preserve a high percentage of living cells and to provide conditions for induction of p73 protein expression, cells were treated transiently with cisplatin. To this end, U373MG cells were treated with 25 μmol/L cisplatin for 2 hours, washed, left for 24 hours, and incubated for further 24 hours with either TNF-α, or TNF-related apoptosis inducing ligand (TRAIL) or 7C11, an agonistic anti-Fas antibody. Apoptosis was then assessed by flow cytometry of Annexin V/propidium iodide double-labeled cells. Figure 1*A* shows that overall, U373MG cells were not constitutively sensitive to 7C11 and to the death ligands tested in the present set of experiments. In contrast, pretreatment with cisplatin rendered cells sensitive to death by 7C11, TRAIL and to a lesser extent by TNF-α compared with mock-treated cells. From this step, further experiments focused mainly on sensitivity of cells to 7C11. As shown in Fig. 1*B*, the percentage of apoptotic Annexin V–positive cells within cisplatin-treated ones dramatically increased following incubation with 7C11 (85.59% versus 21.01%). These data show that treatment of U373MG cells with cisplatin improved their sensitivity to 7C11 and to death ligands suggesting that p73 activation could take part to sensitization process. It is unlikely that in U373MG autocrine or paracrine mechanisms as described in ref. (10) could take part in sensitization to death ligands because no significant apoptosis was observed in the absence of 7C11.

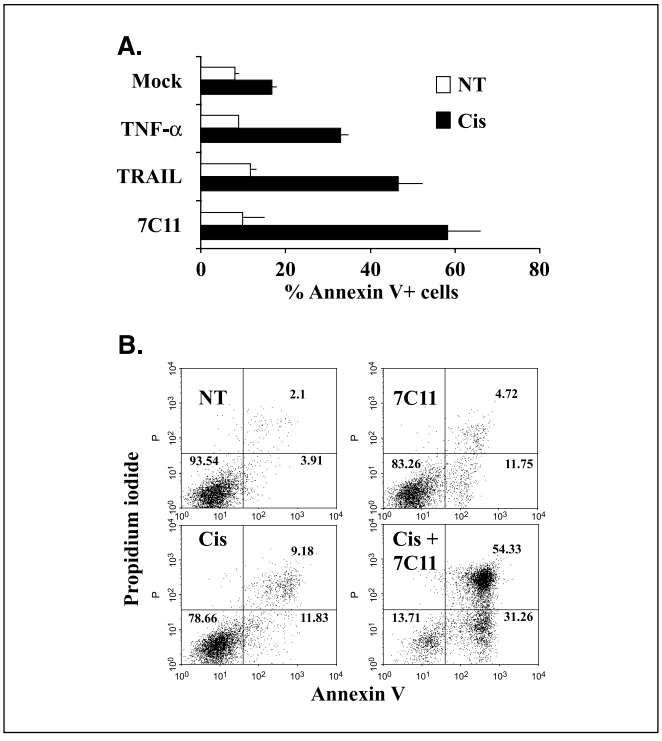

Figure 1. Cisplatin induces sensitization to proapoptotic ligands of the TNF family. U373MG cells were transiently treated (cisplatin, *Cis*; *black columns*) or not treated (*NT*, *white columns*) with cisplatin (25 μmol/L for 2 hours). Cells were then washed and incubated 24 hours later with TRAIL, TNF-α, or with an agonist of Fas (7C11) for an additional period of 24 hours. Apoptosis was quantified using cytofluorimetric analysis of Annexin V and propidium iodide double-labeled cells (*A* and representative picture in *B*). *Columns,* means from three different experiments; *bars,* ±SDs.

Sensitization to death ligands depends on p73 activity. Because we previously showed that the effect of cisplatin on U373MG depended on p73 activity (11), we assessed whether it was involved in sensitivity of cisplatin-treated cells to 7C11. Ensured that cisplatin treatment allowed induction of p73 expression (Fig. 2*A*), cells were infected with an adenovirus recombinant for the dominant-negative isoform ΔN-p73α to prevent p73 activity before incubation with cisplatin and 7C11. Figure 2*B* shows a significant decrease of the percentage of Annexin V^+ in ΔN-p73α infected cells compared with those infected with mock adenovirus, definitely demonstrating requirement of p73 activity for sensitization to 7C11.

To get direct proof that p73 could induce sensitivity to 7C11, p73 was ectopically expressed in U373MG and in p73-negative SK-N-AS cells before incubation with the antibody. To this end, cells were infected with a p73α-recombinant adenovirus (Ad-p73α) and treated with 7C11. Analysis of cell death by trypan blue exclusion and of apoptosis by Western blotting of PARP cleavage (Fig. 2*C*) showed a sensitization of Ad-p73α-infected U373MG to 7C11 and to TRAIL (data not shown) and to a lesser extent to TNF-α. The same effect was observed and even at a higher magnitude for TNF-α in p73−/p53+ SK-N-AS cells, providing further evidence that p73 expression was sufficient to trigger death receptor signaling under treatment with ligands.

p73-dependent apoptosis through Fas depends on caspases activation but is not due to variations in expression of the death-inducing signaling complex components. Resistance of cells to apoptosis can occur through various mechanisms that interfere at different levels of apoptotic pathways. Therefore, it is conceivable that sensitization of resistant cells could result from either blockage of antiapoptotic proteins or activation of proapoptotic ones. Activation of death receptors leads to recruitment of the initiator caspase 8 which then engages executioner caspase 3, an essential step to end up in cell apoptosis. Several mechanisms have been reported in various models to explain how cisplatin can trigger Fas signaling: (i) up-regulation of Fas (10); (ii) activation of c-jun-NH_2-kinase and caspases pathways (19, 20); and (iii) down-regulation of c-FLIP (21, 22), a short-lived cytoprotective protein blocking the initiation of caspase 8 activation at the DISC level. We first assessed whether 7C11-induced apoptosis of cisplatin-treated cells involved caspase 3 activation, by quantifying activated caspase 3 in permeabilized cells by using specific FITC-conjugated mAb and flow cytometry. Figure 3*A* shows constitutive resistance of cells to 7C11 contrasting with caspase 3 activation in 40% of the cells that were pretreated with cisplatin for 24 hours. Use of nonselective (ZVAD), caspase 8 (ZIETD)-, and caspase 9 (ZLHED)-specific inhibitors showed that p73-dependent sensitivity of U373MG cells to 7C11 involved caspase 8 and caspase 9 activation (Fig. 3*B*). Because no cleavage of procaspase 8 (FLICE) was observed in cells treated with 7C11 (Fig. 3*C*), we excluded that constitutive resistance of U373MG cells to 7C11 was due to blockage of Fas signaling downstream of FLICE activation. In contrast, procaspase 8 was undetectable after pretreatment of cells with cisplatin corroborating with an increased number of activated caspase 3–positive cells.

Initiation of death receptor–mediated apoptosis requires assembling of the DISC that results from interactions between receptor death domains of Fas, FADD, and FLICE whose recruitment and activation are essential for death signal to progress. We then examined expression of FADD and FLICE

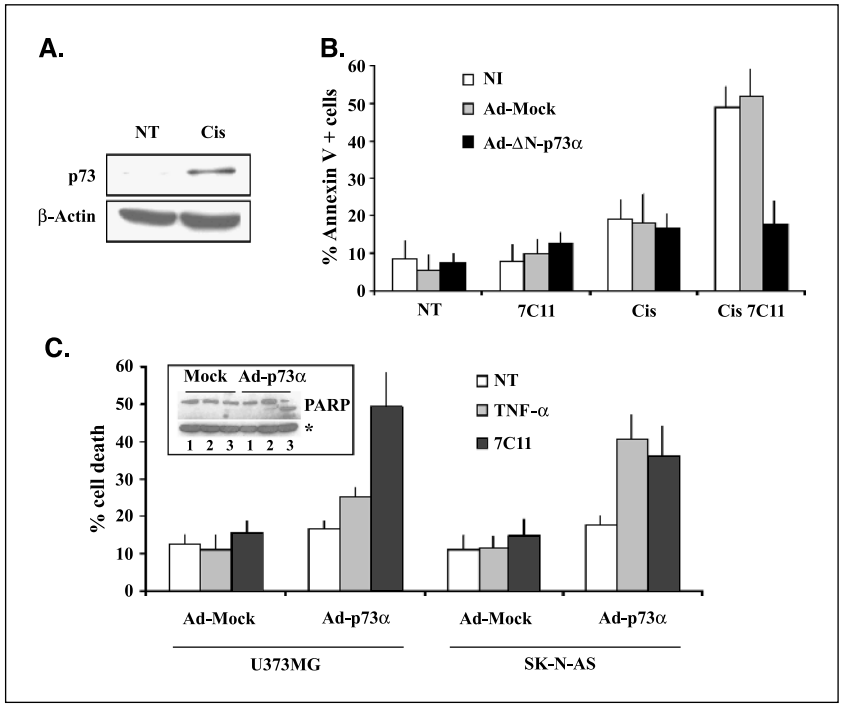

Figure 2. Sensitization of death receptors depends on p73 activity. *A,* cells were transiently treated (cisplatin, *Cis*) or not (*NT*) with cisplatin, washed, and analyzed 24 hours later using Western blotting for the expression of p73. U373MG cells were preinfected (MOI of 50 for 24 hours) with an adenovirus recombinant for ΔN-p73α (Ad-ΔN-p73α) or an empty adenovirus (Ad-Mock) or not infected (*NI*) before treatment with cisplatin (25 μmol/L for 2 hours) and 7C11 24 hours later. *B,* % apoptotic Annexin V^+ cells was determined 24 hours later by flow cytometry. U373 MG (p73+) or SK-N-AS (p73−) cells were infected with Ad-p73α or Ad-Mock as described above, then incubated for 24 hours with TNF-α (*hatched columns*) or 7C11 (*black columns*). *C,* cell death was analyzed by counting trypan blue–colored cells. Apoptosis was analyzed using Western blotting of PARP cleavage and of β-Actin as a control (*, *inset* in *C*: *lane 1,* NT; *lane 2,* TNF-α; *lane 3,* 7C11). *Columns,* means from three different experiments; *bars,* ±SDs.

by Western blotting under treatment with cisplatin or ectopic overexpression of p73α. No detectable variations were observed in FADD and FLICE expression in cisplatin-treated as well as in Ad-p73α-infected cells compared with untreated, uninfected and mock-infected (Ad-Mock) ones (Fig. 3*D*). According to the known inhibition of procaspase 8 recruitment to the DISC by high levels of c-FLIP as a major blocking mechanism (23), cells were checked for the expression of c-FLIP-S and c-FLIP-L isoforms. No detectable change in c-FLIP expression was observed on Western blots of both cisplatin-treated cells and those infected with Ad-p73α compared with controls (Fig. 3*D*). Altogether, these data showed that p73-dependent sensitivity of U373MG cells to 7C11 depends on caspases activation and was unlikely due to variations in expression of the DISC components FADD, FLICE and c-FLIP.

Sensitization to 7C11 depends on p73 transcriptional activity and on increased expression of surface Fas. Because cross-talk between p53 and death receptors to initiate apoptosis

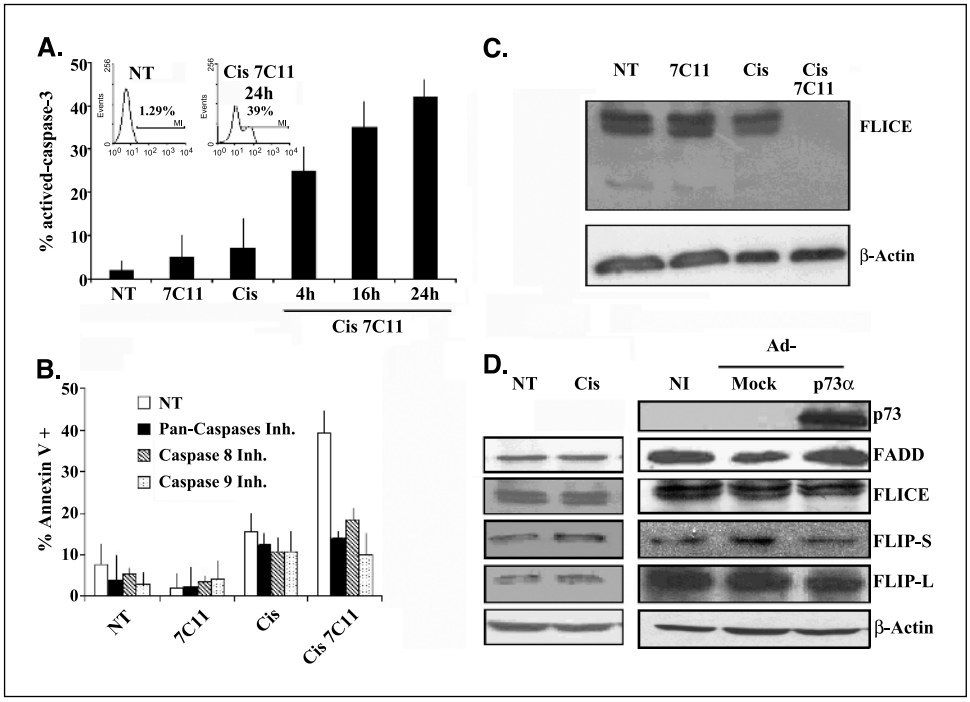

Figure 3. Sensitization to 7C11 induced by cisplatin requires caspase activation and induces no change in expression of the DISC proteins. U373MG cells were treated or not with cisplatin (25 μmol/L, 2 hours) and incubated 24 hours later with 7C11 for 4, 12, or 24 hours. *A,* caspase 3 activation was analyzed using cytofluorimetric analysis with an antibody specific for activated caspase 3. U373MG cells were preincubated for 6 hours with pan-caspases, caspase 8, or caspase 9 inhibitors as indicated and treated with cisplatin and with 7C11 as described above. *B,* apoptosis was quantified 24 hours later using cytofluorimetric analysis of Annexin V–stained cells. U373MG cells were treated or not with cisplatin (25 μmol/L, 2 hours) and incubated 24 hours later with 7C11 for 12 hours. *C,* β-actin expression and FLICE cleavage were analyzed using Western blotting with specific antibodies. *D,* expression of p73, β-actin, and of the DISC proteins (FADD, FLICE, c-FLIP-S, and c-FLIP-L) was analyzed on nontreated (*NT*), cisplatin-treated (*Cis*), noninfected (*NI*), Ad-Mock-, and Ad-p73α-infected cells (MOI of 50 for 24 hours) using Western blotting with specific antibodies. *Columns,* means from three different experiments; *bars,* ±SDs.

involves both transcription-dependent and -independent pathways, we may ask whether sensitization of U373MG cells depends on p73 transcriptional activity. To address this question, cells were infected, before incubation with 7C11, with a recombinant adenovirus Ad-p73M293 expressing a mutant of the DNA binding domain and known to confer loss of transcriptional activity (3). Figure 4*A* shows that ectopic expression of p73M293 did not sensitize cells to 7C11 contrary to p73α, suggesting that binding of p73 to specific target promoters was required for sensitization to 7C11. To assess whether Fas transcription could be increased under ectopic overexpression of p73α, reverse transcription-PCR (RT-PCR) analysis was done on cells infected with the above-mentioned recombinant adenoviruses. Figure 4*B* shows an increased transcription of Fas in cells expressing p73α contrary to those expressing p73M293, demonstrating that *Fas* gene could be a target of p73. Western blot analysis of cells infected with mock or recombinant adenovirus shows a significant increase in total expression of Fas (Fig. 4*C*, 5-fold increase versus baseline amount) in cells overexpressing p73α contrary to those expressing the M293 mutant. Finally, to assess whether up-regulation of Fas expression was found at the cell surface, cytofluorimetric analyses were done on cells either treated with cisplatin or infected with adenoviruses. Figure 4*D* confirms an increased expression of Fas at the cell surface following both cisplatin treatment and ectopic overexpression of p73α.

Altogether these data showed that in U373MG cells, activation of p73 transcriptional activity made cells sensitive to 7C11 through mechanisms including up-regulation of Fas expression at the cell surface.

Human cytomegalovirus infection inhibits p73-dependent Fas-mediated apoptosis. We showed in a previous study that HCMV-infected astrocytoma and neuroblastoma cells became resistant to p73- and p53-dependent apoptosis because of an overexpression of the dominant-negative isoform ΔN-p73α. Interestingly, expression of this antiapoptotic isoform in tumor cells (24, 25) calls the question of how HCMV could contribute to resistance to drugs acting through death receptors. In accordance with data in Fig. 2*B* showing that ΔN-p73α could inhibit p73-dependent activation of the Fas pathway, we determined whether HCMV infection could interfere with sensitivity of cisplatin-treated cells to 7C11. Figure 5*A* shows that infection with HCMV, before cross-linking with 7C11 antibodies, inhibited Fas-mediated cell

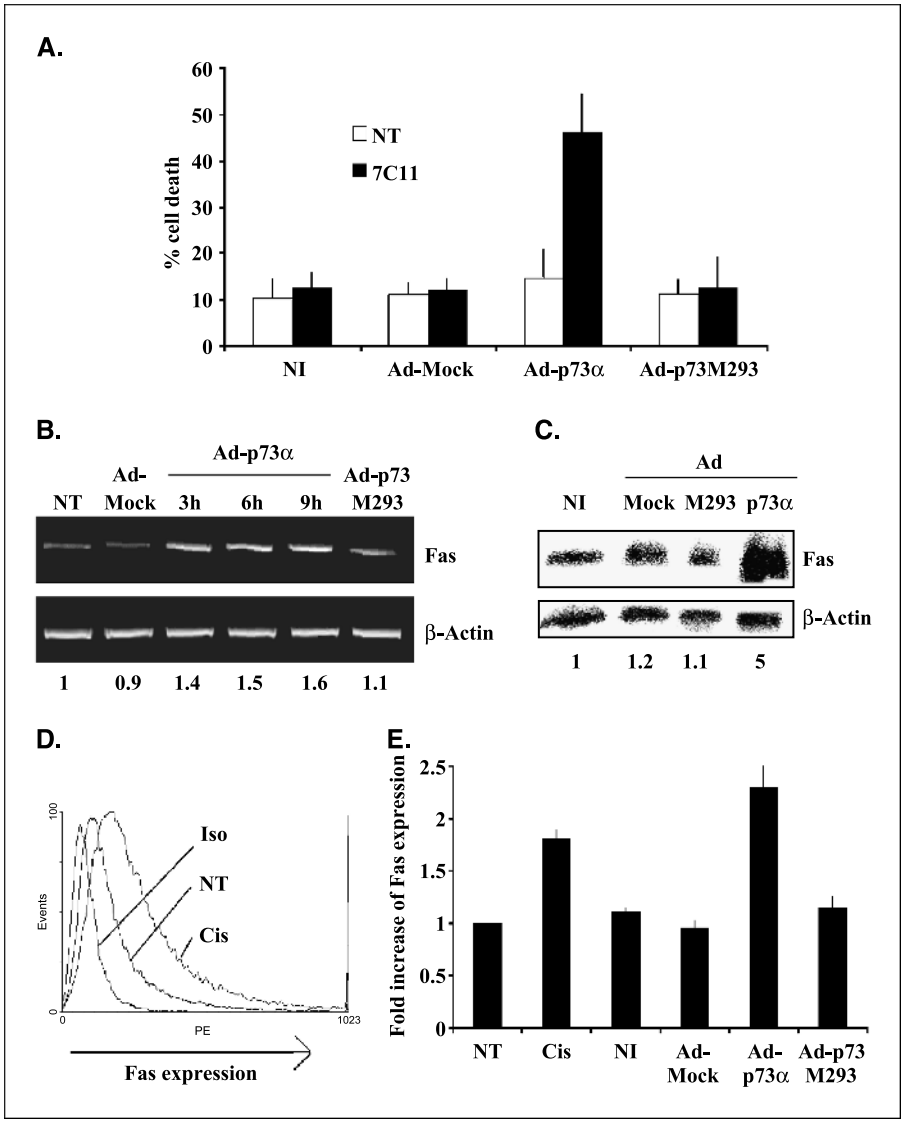

Figure 4. p73-transcriptional activity is required for sensitization to 7C11 and induces an increased transcription and expression of Fas. U373MG cells were infected with Ad-Mock, Ad-p73α, or an adenovirus recombinant for a transcriptionally inactive mutant of p73α (Ad-p73M293; MOI of 50). *A,* cells were incubated 24 hours later with 7C11 for an additional 24 hours and cell death was analyzed by counting trypan blue-colored cells. U373MG cells were treated with cisplatin (*Cis*) or not treated (*NT*), infected with Ad-Mock, Ad-p73α, Ad-p73M293 (MOI of 50) or not infected (*NI*). *D,* cell surface expression of Fas was analysed 24 hours later using cytofluorimetry with specific or isotypic control (*Iso*) mAbs (*left*). Fold increase of Fas expression was calculated from mean fluorescence relative to those of nontreated (*NT*) or noninfected cells (*NI*) cells (*right*). U373MG cells that were infected with Ad-Mock, Ad-p73α, or Ad-p73M293 (MOI of 50) were analyzed for Fas and β-actin transcript expression by RT-PCR using specific primers (*B*) and for protein expression by Western blotting (*C*). Quantitative display of Fas transcripts and protein expression relative to β-actin in B and C. *Columns,* means from three different experiments; *bars,* ±SDs.

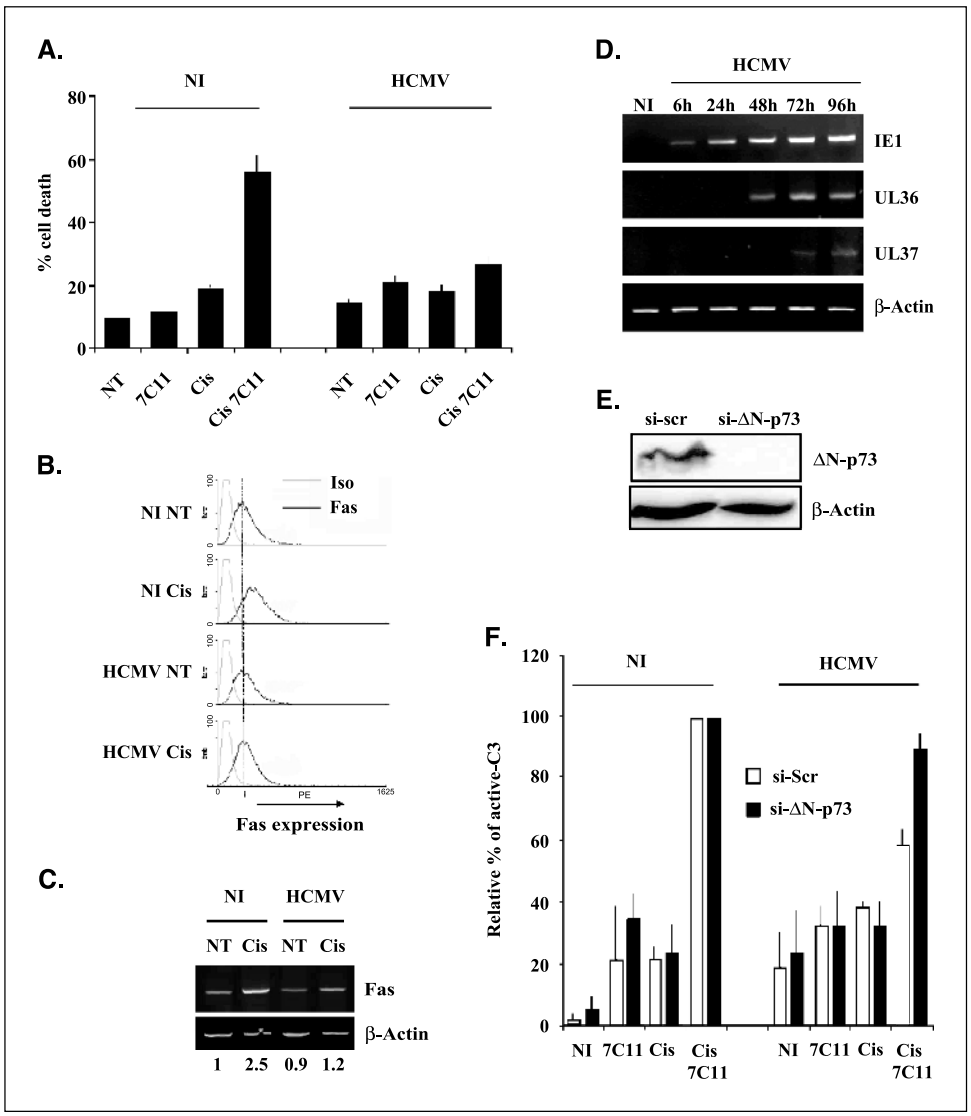

Figure 5. HCMV-infected cells are resistant to p73-induced sensitization to death ligands. U373MG cells were infected with HCMV (strain AD169, MOI of 1) for 48 hours or not infected (*NI*). Cells were then treated with cisplatin (*Cis*, 25 μmol/L for 2 hours) or not treated (*NT*) and incubated 24 hours later with or without 7C11 for an additional period of 24 hours. *A,* cell death was analyzed by counting trypan blue-colored cells. *B,* alternatively, cells were transiently treated with cisplatin (*Cis*) for 24 hours or not treated (*NT*) and analyzed for Fas expression on cell surface using cytofluorimetry with a specific antibody or an isotypic control (*ISO*). U373MG cells well noninfected (*NI*) or infected with HCMV (see above) treated or not (*NT*) with cisplatin (*Cis*) and analyzed by RT-PCR for the expression of Fas mRNA. C, ratios of Fas transcripts expression to those of β-actin are as indicated. U373MG cells were infected with HCMV for various time periods (6, 24, 48, 72, and 96 hours; MOI of 1). *D,* expression of transcripts for IE1, UL36, UL37, and β-actin was analyzed using RT-PCR with specific primers. U373MG cells were stably transfected with scramble (si-scr) or ΔN-p73α (si-ΔN) silencing RNA and infected with Ad-ΔN-p73α (MOI of 0.5). ΔN-p73α expression level was checked using Western blotting. *E,* β-actin band served as internal control. Si-scr- and si-ΔN-transfected U373MG cells were infected with HCMV (strain AD169, MOI of 1 for 48 hours) or not infected (*NI*). Cells were then treated with cisplatin and 7C11 as described above. *F,* apoptosis was quantified using the caspase 3 ApoAlert colorimetric assay kit. *Columns,* means from four different experiments; *bars,* ±SDs.

death of cisplatin-treated cells that may be due to the suppressive activity of ΔN-p73α. Ensured that all the cells were infected by HCMV, expression of Fas was analyzed by cytofluorimetry following treatment with cisplatin. Histograms of Fig. 5*B* showing mean fluorescence of labeled cells reveal that HCMV infection inhibited cisplatin-induced up-regulation of Fas. Analyses of Fas mRNA expression by RT-PCR further showed that the viral effect appeared at the transcriptional level because cisplatin-mediated up-regulation of Fas transcription was no longer recovered after HCMV infection (Fig. 5*C*). These data suggest that resistance to apoptosis by 7C11 was due to an insufficient amount of Fas molecules on the cell surface that may result from blockage of p73 transcriptional activity. At this stage, we examined expression of UL36 and UL37 viral gene products that are known to exert inhibitory activities on apoptosis of HCMV-infected cells. RT-PCR analyses of viral transcripts throughout infection of U373MG cells showed that in the present conditions of infection, UL36 and UL37 mRNA were detected at 48 and 72 hours post-infection, respectively (Fig. 5*D*). Although kinetics of UL36 and UL37 expression differ in our experiments from those previously described by others, their effect may depend on a threshold level of protein expression with respect of cell type as well as of multiplicity of infection used in experiments. Anyway, our data do not exclude that besides ΔN-p73α, these proteins could take part to blockage of apoptosis at both the DISC and mitochondria levels.

To further address whether HCMV could contribute to inhibition of Fas apoptosis through overexpression of ΔN-p73α, cells were stably transfected with small interfering RNA either scrambled (si-scr) or targeted specifically against the ΔN-isoform (si-ΔN-p73α). Figure 5*E* shows that expression of ΔN-p73α could be switched off in U373MG cells demonstrating the efficiency of ΔN-p73 interfering RNA. Then, si-ΔN-transfected cells were either mock-infected or infected with HCMV, treated or not with cisplatin and submitted or not to incubation with 7C11. Figure 5*F* reveals that within mock-infected cells both si-scr- and si-ΔN-transfected ones were highly sensitive to 7C11 (100% apoptosis). Among si-scr-transfected cells, HCMV infection made cells less sensitive to 7C11 but at a lesser extent within scr-transfected cells (50% versus 100%) than within si-ΔN-transfected ones (85% versus 100%). These data suggest that in cisplatin-treated cells ΔN-p73α activity takes part in HCMV-induced inhibition of apoptosis by 7C11.

Discussion

Although our knowledge of molecular mechanisms involved in p73-dependent DNA damaging pathway are in progress, it is still unknown whether p73 could take part to regulation of death receptor signaling. According to the ability of p53 to participate in death receptor–mediated apoptosis and in immune surveillance of tumors by cytotoxic effector cells, we may ask whether p73 could compensate for p53 deficiency. Moreover, the description of p73 isotypes with deleted transactivation domain (ΔTA) and findings showing that the ratio of TA to ΔTA isoforms expression was a major regulatory mechanism of p53 and p73 activities, made this matter even more relevant. To address this question, we used the p73+/p53− astrocytoma cell line U373MG, that is known to be constitutively resistant to death ligands but sensitive to the DNA damaging agent cisplatin in a p73-dependent manner.

In this report, we showed that p73 can sensitize to apoptosis through death receptors in a transcription-dependent manner, leading to apoptosis of target through a pathway depending on caspase activation. We found that sensitization of cisplatin-treated targets through Fas was not due to variations in expression of the DISC components FADD, FLICE, and c-FLIP, but that Fas transcription was increased with consequent up-regulation of protein expression at the cell surface. In accordance with the ability of HCMV to flood infected cells with the ΔN isoform (11), we showed that infection takes part in inhibition of p73-dependent sensitization to Fas apoptosis as shown by using si-ΔN-p73α-transfected cells. Our data provide evidence that p73 can exert p53-like activities such as up-regulation of Fas transcription and protein expression and compensate for p53 deficiency, a permanent feature in half of tumors. Although our data show that p73 can drive *Fas* gene transcription, we cannot exclude that triggering of other effector proapoptotic genes could take part to the process. We showed that in U373MG cells, p73-dependent sensitization to Fas was dependent on caspase 9 activation, classifying these cells as type II (26), known to use mitochondria as amplifier of the apoptotic signal. Whether p73 could target proapoptotic mitochondrial mediators in these cells remains an open question.

It has been shown that HCMV can block Fas signaling through inhibition of caspase 8 by the viral gene product UL36 (13) and through interference with the mitochondrial pathway by UL37, a viral protein that mimics the antiapoptotic activity of Bcl-2 (12). It is most unlikely that UL36 protein could exert its inhibiting activity in U373MG cells because *UL36* gene is mutated in AD169 strain used in our study (12) and thus cannot exert blockage of the DISC. In contrast, we cannot exclude that UL37 could take part to blocking of Fas signal in the late phase of infection, providing an additional mechanism whereby HCMV may suppress mitochondrial activity in p73-positive cells. This could explain why ectopic overexpression of si-ΔN-p73α partially restored sensitivity to 7C11 in HCMV-infected cells. The combined antiapoptotic activities of UL37 and ΔN-p73α could reinforce harmfulness of HCMV in events such as neurogenesis where mitochondria are central components. Therefore, by increasing its means of action against the host, HCMV is armed with new weapons to favor its survival in both type I and type II cells.

The outcomes of p73 activity on Fas signaling are numerous because this death receptor takes part to multiple processes including development of the central nervous system, immune surveillance of neural cells, and sensitivity of tumors to drugs. The potential role of Fas in the active killing of neurons and following stress has been suggested recently (27) which may call up p73 activity. It has also been shown that Fas-dependent apoptosis constitutes a prominent mechanism for tumor clearance by cytotoxic T cells *in vivo* (28). In conclusion, we may suggest that in addition to its role in the developing brain (5), p73 activity could take part to surveillance against tumors and viruses through its connection with death receptors.

Some antitumor drugs are known to act through p53-dependent up-regulation of either death receptors or death ligands, or both, as previously reported for Fas and FasL in chemosensitive neuroblastoma (10). It is unlikely that in U373MG cells, p73-induced apoptosis could be triggered through such an autocrine or paracrine pathway because both treatment with cisplatin and infection with Ad-p73α by their own did not increase significantly the percentage of apoptotic cells compared with untreated cells.

Wherever p73-dependent death receptor signaling could take place, either in development and immune surveillance of central nervous system or in surveillance and treatment of tumor, we expect that up-regulation of the dominant-negative isoform ΔN-p73α could impair all these running processes. According to the present data and to our previous work demonstrating a major role of HCMV in blocking p53- and p73-dependent DNA damaging in infected cells, we consider that HCMV may interfere with p73- and p53-dependent death receptors signaling in all the above mentioned physiologic processes, through up-regulation of ΔN-p73α.

Altogether our data give the first evidence that p73 functions could be required to switch on physiologic processes depending on death receptors signaling. Overall, our previous (11) and present data and moreover, recent findings showing that on the one hand, ΔN-p73α isoform is up-regulated in human tumors (25, 29) and on the other hand HCMV infection and expression is associated with malignant glioma (30) and colorectal cancers (31) prompt us to consider accumulation of ΔN-p73α as a major mechanism in oncomodulation by HCMV.

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References

1. Balint EE, Vousden KH. Activation and activities of the p53 tumour suppressor protein. Br J Cancer 2001;85:1813–23.
2. Zhu K, Wang J, Zhu J, et al. p53 induces TAP1 and enhances the transport of MHC class I peptides. Oncogene 1999;18:7740–7.
3. Kaghad M, Bonnet H, Yang A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997;90:809–19.
4. Yang A, Kaghad M, Wang Y, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998;2:305–16.
5. Yang A, Walker N, Bronson R, et al. p73-Deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 2000;404:99–103.
6. Yang A, Kaghad M, Caput DMcKeon F. On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet 2002;18:90–5.
7. Sheikh MS, Burns TF, Huang Y, et al. p53-Dependent

and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor α. Cancer Res 1998;58:1593–8.
8. Zhu J, Jiang J, Zhou W, Chen X. The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res 1998;58:5061–5.
9. Lee CW, La Thangue NB. Promoter specificity and stability control of the p53-related protein p73. Oncogene 1999;18:4171–81.
10. Fulda S, Sieverts H, Friesen C, Herr I, Debatin KM. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res 1997;57: 3823–9.
11. Allart S, Martin H, Detraves C, et al. Human cytomegalovirus induces drug resistance and alteration of programmed cell death by accumulation of δN-p73α. J Biol Chem 2002;277:29063–8.
12. Goldmacher VS, Bartle LM, Skaletskaya A, et al. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A 1999;96:12536–41.
13. Skaletskaya A, Bartle LM, Chittenden T, et al. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc Natl Acad Sci U S A 2001;98:7829–34.
14. Reddehase MJ. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat Rev Immunol 2002;2:831–44.
15. Tan JT, Whitmire JK, Murali-Krishna K, et al. 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination. J Immunol 2000;164:2320–5.
16. Gong JG, Costanzo A, Yang HQ, et al. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature 1999;399:806–9.
17. Wu GS, Burns TF, McDonald ER III, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997;17:141–3.
18. El-Deiry WS. Insights into cancer therapeutic design based on p53 and TRAIL receptor signaling. Cell Death Differ 2001;8:1066–75.
19. Sanchez-Perez I, Murguia JR, Perona R. Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 1998;16:533–40.
20. Koo MS, Kwo YG, Park JH, et al. Signaling and function of caspase and c-jun N-terminal kinase in cisplatin-induced apoptosis. Mol Cells 2002;13:194–201.
21. Fulda S, Meyer E, Debatin KM. Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. Cancer Res 2000;60:3947–56.
22. Kinoshita H, Yoshikawa H, Shiiki K, et al. Cisplatin (CDDP) sensitizes human osteosarcoma cell to Fas/CD95-mediated apoptosis by down-regulating FLIP-L expression. Int J Cancer 2000;88:986–91.
23. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J Biol Chem 2001;276:20633–40.
24. Casciano I, Mazzocco K, Boni L, et al. Expression of ΔNp73 is a molecular marker for adverse outcome in neuroblastoma patients. Cell Death Differ 2002;9:246–51.
25. Douc-Rasy S, Barrois M, Echeynne M, et al. ΔN-p73α accumulates in human neuroblastic tumors. Am J Pathol 2002;160:631–9.
26. Scaffidi C, Schmitz I, Zha J, et al. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. J Biol Chem 1999;274:22532–8.
27. Raoul C, Pettmann B, Henderson CE. Active killing of neurons during development and following stress: a role for p75(NTR) and Fas? Curr Opin Neurobiol 2000;10:111–7.
28. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R. Immune escape of tumors *in vivo* by expression of cellular FLICE-inhibitory protein. J Exp Med 1999;190: 1033–8.
29. Zaika AI, Slade N, Erster SH, et al. ΔNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J Exp Med 2002;196:765–80.
30. Cobbs CS, Harkins L, Samanta M, et al. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res 2002;62:3347–50.
31. Harkins L, Volk AL, Samanta M, et al. Specific localisation of human cytomegalovirus nucleic acids and proteins in human colorectal cancer. Lancet 2002;360:1557–63.