Human Cytomegalovirus Induces Drug Resistance and Alteration of Programmed Cell Death by Accumulation of ΔN-p73α

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Intrauterine transmission of human cytomegalovirus (HCMV) to the fetus following primary infection in early and late pregnancy usually results in severe neurological handicaps and sensorineural hearing loss with typical migrational anomalies, optic atrophy, disturbed myelination, cerebellar hypoplasia, microcephaly, hydrocephaly, and lissencephaly. Recently, evidences raised from the phenotype of p73-deficient mice show that an association may exist between the expression of the TP53 homologous gene and HCMV tropism in the brain, suggesting an implication of p73 in viral persistence. In this study, we demonstrated that HCMV-mediated inhibition of apoptosis only occurs in p73-expressing cells. Upon infection, an accumulation of ΔN-p73α isoforms was observed in HCMV-infected p73-positive cells. This phenomenon was shown to be responsible for the subsequent acquired resistance to apoptosis of infected cells. Inhibition of apoptosis in p73-positive cells by HCMV may thus contribute both to virus persistence and abnormal nervous cell survival. This finding provides the first molecular basis for HCMV-associated abnormal embryonic development and neurological defects in newborns.

Human cytomegalovirus (HCMV), a β-herpes virus, is harmless to most immunocompetent people. However, HCMV is responsible for serious illness and death in immunocompromised hosts such as AIDS patients, as well as patients receiving immunosuppressive treatment following organ transplantation. Cytomegalovirus infection is the most common congenital viral infection and carries a high risk of long term morbidity and mortality. Intrauterine transmission to the unprotected fetus in early and late pregnancy usually results in death or in severe neurological defects including sensorineural hearing loss, optic atrophy, disturbed myelination, cerebellar hypoplasia, microcephaly, hydrocephaly, and lissencephaly (for reviews, see Refs. 1 and 2). How morphological and functional disorders develop is unclear since little is known about mechanisms underlying the pathogenicity of the virus.

Apoptosis is an important process participating in the formation of organs and tissues during embryogenesis and in the clearance of abnormal or misfunctioning cells in the body. At any stages of life, interference with this process is predicted to be damaging for organisms. In the mouse developing brain, surprisingly, murine cytomegalovirus (mCMV) was found to induce apoptosis in non-infected cells, whereas a blocking of apoptosis occurred in infected mature neurons (3). Similar results were obtained with human neuroblastoma cells, which become “immortalized” and resistant to cytotoxic stress following HCMV infection (4). As with many other viruses, HCMV has developed strategies to inhibit apoptosis of infected cells, thus escaping from immune clearance, promoting abnormal cell survival, and favoring viral persistence. Some reports discuss possible mechanisms by which HCMV can block apoptosis. HCMV proteins can directly interact with the tumor suppressor p53 and interfere with either cell cycle arrest or apoptosis (5–10). The infection can also induce a “nuclear exclusion” of p53 (11, 12), a previously described process responsible for p53 inhibition (13). In addition to p53 inactivation, HCMV can block apoptosis by inhibiting caspase-8 (Flice) (14) or by mimicking the antiapoptotic activity of Bcl-2 (15). As pointed out above, HCMV antiapoptotic activities may account for virus persistency and pathogenicity.

Recently, a p53 homologue termed p73 was described (16), and evidence was raised from the phenotype of p73-deficient mice that p73-expressing cells such as Cajal-Retzius cells, vomeronasal organ neurosensory neurons, (17), and sympathetic neuroblasts (18) were lost in the mutant. These observations suggested that functional p73 was required for neuron survival.

The degree of overlaps between cytomegalovirus tissue tropism and p73-positive cell distribution in brain, as noticed early on, suggested a link between viral persistence and p73 gene products. The discovery of p73 dominant negative isoforms thus prompted us to assess a possible relationship between HCMV antiapoptotic activities and p73 expression in infected cells. We hypothesized that one mechanism by which the virus may protect cells against apoptosis was by up-regulating endogenous inhibitors of p53 and 73 such as ΔN-p73. Our results demonstrate (i) that the blockage of apoptosis by HCMV relies on p73 expression, (ii) that HCMV, indeed, induces a robust stabilization of ΔN-p73 protein and not of p73, and (iii) finally, that the up-regulation of ΔN-p73 is per se sufficient to inhibit the apoptotic process.

EXPERIMENTAL PROCEDURES

Cell Cultures—IMR-32 and SK-N-AS neuroblastoma and U373MG astrocytoma cell lines were from the American Type Culture Collection (ATCC). IMR-32 and SK-N-AS were maintained in Dulbecco’s modified Eagle’s medium and U373MG in RPMI medium (Invitrogen), both containing 10% fetal calf serum (Invitrogen), and supplemented with 10
ΔN-p73α Protects HCMV-infected Cells from Apoptosis

Fig. 1. HCMV infection inhibited sensitivity to cisplatin-induced apoptosis in U373MG cells (p73+ /p53−). U373MG cells were either mock-infected (−) or infected (+) for 1, 2, or 3 days (d1, d2, d3) with HCMV at a m.o.i. of 3. Then, cells were treated (+) or not treated (−) with cisplatin (cis) at 250 μM for an additional 48 h at different times p.i. The indicated times p.i. do not include cisplatin treatment. Cell death was quantified by counting trypan blue-colored cells (a). Apoptosis was analyzed by Western blotting of PARP cleavage (b) and measured by cytofluorimetric analysis of annexin V-propidium iodide double-labeled cells (c). Permeabilized U373MG cells were labeled with rabbit anti-p73 antibodies and examined on a light fluorescent microscope (d).

μg/ml Oflocet anti-mycolplasma (Rousell, Paris, France) and sodium pyruvate unless otherwise stated. Transcript sequences and protein expression of p73 and p53 have been described in Ref. 16: IMR-32 express wild-type p53 and p73 (p53WIt, p73S); SK-N-AS express p53S but undetectable levels of p73 (p53S, p73S); and U373MG express wild-type p73 and mutant p53S (p73S, p53WIt). Viruses—HCMV AD169 (ATCC) was propagated in human foreskin fibroblasts in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Viruses were collected, and total proteins were quantified using a Bio-Rad protein assay kit (Bio-Rad). 30 μg of proteins were boiled for 5 min in 5% β-mercaptoethanol reducing Laemmli sample buffer, and samples were separated by SDS-PAGE in 12.5% denaturing polyacrylamide gels and transferred to nitrocellulose membrane (Hybond C, Amersham Biosiences). Immunoblots were stained with Ponceau red to visualize total proteins contained in each slot and probed alternatively with the following antibodies: rabbit polyclonal anti-human p73S (16) used at 1/2000 dilution; E13, a mouse monoclonal antibody specific for HCMV immediate-early (IE) proteins (E1E; UL122) and E2E (UL123), hybridoma supernatant provided by M. Mazeron, Paris, France) at 1:10 dilution; PARP mouse monoclonal antibody (1:10,000 dilution, CLONTECH laboratories); or a human p21 (Cip1/WAF1) mouse monoclonal antibody (1: 500 dilution, BD Transduction Laboratories). Blots were revealed using peroxidase-conjugated polyclonal anti-rabbit antibody (1:5000 dilution, BD Transduction Laboratories) or peroxidase-conjugated polyclonal anti-mouse antibody (1:10,000 dilution, Sigma) and ECL detection kit (Amersham Biosiences). 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: p73S sense (exon 1), 5'-GGGGCAGCGAGCGAACC-3'; p73S antisense (exon 10), 5'-CCATAGCTCCAGGCTCTC-3'; ΔN-p73S sense (exon 3), 5'-ACAACGCGCCGGCATTC-3'; ΔN-p73S antisense (exon 10), 5'-CCATAGCTCCAGGCTCTC-3', with 33 cycles. Coamplification of GAPDH was used as an internal control.

Immunofluorescence Staining—U373MG cells were seeded on Labtek chamber slides (Nunc, Naperville, IL) at 1.5×10^4 cells/well. Cells were fixed for 1 h in PBS containing formaldehyde (3.7% final), washed in PBS, and then permeabilized in methanol/acetone (1:1) at 37 °C in the above conditions, cells were washed with PBS and detached with trypsin. Cell death was detected by counting the trypan blue-(Invitrogen) colored cells. The percentage of dead cells was calculated as follows: (number of colored dead cells/number of total cells)×100. The presence of apoptotic cells was also detected by multiparameter flow cytometry (Coulter Epics) using propidium iodide and fluorescein isothiocyanate-conjugated annexin V (Coulter-Immunotech) double labeling according to the manufacturer’s instructions and by Western blotting of PARP cleavage as described below.

Western Blotting—At different times post-infection, monolayers of U373MG, IMR-32, or SK-N-AS cells were harvested, washed, and either stocked at −20 °C or immediately lysed by incubation for 5 min on ice in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% desoxycholate, 0.1% SDS, pH 8). Lysates were homogenized with a pipette and centrifuged for 15 min at 10,000 rpm. Supernatants were collected, and total proteins were quantified using a Bio-Rad protein assay kit (Bio-Rad). 30 μg of proteins were boiled for 5 min in 5% cell debris by centrifugation at 1,500 × g for 10 min at 4 °C and were stored at −70 °C until use. Virus titers were determined by plaque-forming unit titration in human foreskin fibroblasts according to standard procedures. A multiplicity of infection (m.o.i.) of 3 was used for all the experiments.

Type 5 adenovirus recombinant for p73S (Ad-p73S) or ΔN-p73S (Ad-ΔN-p73S) were obtained as follows: Ad-p73S was produced by homologous recombination in 293 cells after co-transfection of the linearized cDNA under the control of the CMV promoter, the pIX sequence of adenovirus, and the DNA of d1324 digested with the restriction enzyme CciI. β-Galactosidase recombinant virus (Ad-β-galactosidase) was prepared as described (19). Viruses were purified by cesium-chloride centrifugation, aliquoted in 20 μl of phosphate-buffered saline (PBS) containing 10% glycerol, and stored at −80 °C until use. The presence of p73 sequences was tested by restriction enzyme digestion and by sequencing the viral DNA. Optimal m.o.i. to obtain cells infected with recombinant adenoviruses was determined by using Ad-β-galactosidase and blue staining as follows: cells were seeded into a 25-cm² flask and infected with Ad-β-galactosidase as described above at various m.o.i. values. Virus was removed after 3 h, and cells were incubated for a further 48 h. After three washes in PBS, cells were fixed for 5 min in 0.4%M glutaraldehyde, 4% formaldehyde, washed, and incubated at room temperature overnight with PBS containing 4 mM potassium ferricyanide, 0.8 mg/ml X-gal, and 5 mM MgCl₂. Cells were washed and then examined under a light microscope.

Adenoviruses were used at 50 m.o.i. in serum-free medium. Virus was removed after 4 h, and cells were incubated in normal medium for a further 20 h.

Cell Death Assays—U373MG, SK-N-AS, or IMR-32 cells were cultured in six-well culture plates in their respective medium containing 10% fetal calf serum and supplemented with sodium pyruvate and Oflocet, as described above. Cells were either mock-infected or infected with HCMV AD169 as indicated or with adenoviruses (Ad-β-galactosidase, Ad-p73S, or Ad-ΔN-p73S) during 24 h. Cisplatin (Sigma) at 250 μM final concentration was added to cell culture and incubation continued at 37 °C for a further 48 h. IMR-32 cells were treated with actinomycin D (Sigma) at a 2 μM final concentration for 15 h. After incubation at
SK-N-AS cells from apoptosis induced by cisplatin.

Cell lysates were submitted to Western blotting (a) using monoclonal antibodies directed against immediate-early proteins (IE1 and IE2) at days p.i. as indicated (d1–d3). Cell death (b) and apoptosis (c) were quantified using counting of trypan blue-positive cells (b) and Western blotting of PARP cleavage (c), respectively. Mock-infected (d0) and HCMV-infected cells (d3) have been treated (+) or not treated (−) with cisplatin (cis) for 48 h, as indicated.

Expression of ΔN-p73α protein was stabilized in U373MG cells throughout infection with HCMV. U373MG cells were either mock-infected (n.i.) or infected for the indicated times with HCMV (m.o.i. of 3) or with adenovirus recombinant for β-galactosidase (Ad-βgal), p73α (Ad-p73a), or ΔN-p73α (Ad-ΔN-p73α) for 24 h (m.o.i. of 50). Lysates from HCMV-infected cells were submitted to SDS-PAGE and blotted with anti-p73 antibodies (a). RNA was extracted, reverse-transcribed, and submitted to semiquantitative reverse transcription-PCR using specific primers for p73α and GAPDH (b).

Ectopic expression of ΔN-p73α rescued U373MG and SK-N-AS cells from apoptosis induced by cisplatin. U373MG and SK-N-AS cells were infected with adenoviruses recombinant for β-galactosidase (Ad-βgal), p73α (Ad-p73a), or ΔN-p73α (Ad-ΔN-p73α) for 24 h (m.o.i. of 50). Cells were then treated (+) or not treated (−) for 48 h with 250 μM cisplatin (cis). Total cell extracts were submitted to SDS-PAGE and blotted with a mouse anti-PARP monoclonal antibody. Molecular sizes of native and cleaved PARP were as indicated (−).

HCMV Infection Inhibits Sensitivity to Cisplatin-induced Apoptosis in p73α-positive U373MG (p73α+/p53−) but Not in p73α-negative SK-N-AS (p73α−/p53+) Cells—It has been well established that cisplatin exerts its cytotoxic effect through induction of DNA damage and activation of apoptosis, both involving p53 or p73 proteins (21). Sensitivity to cisplatin has been determined in HCMV-permissive U373MG astrocytoma cells that have been selected as they express wild-type p73 (p73+), or mutated p53 (p53−) (16). Fig. 1 shows that treatment with cisplatin for 48 h resulted in cell death, as assessed through trypan blue exclusion, but that cells became resistant following infection by HCMV (Fig. 1a). It is noteworthy that the ratio of infected to uninfected dead cells within cisplatin-treated cells increased dramatically from day 0 (40 versus 60%) to day 2 (75 versus 50%) and day 3 (100 versus 30%), suggesting that HCMV-induced antiapoptotic process was more efficient as infection progressed. Apoptosis was observed by using Western blotting of PARP cleavage (Fig. 1b) and annexin V-propidium iodide double labeling (Fig. 1c), suggesting that in U373MG cells, p73 contributed to cisplatin-induced programmed cell death. In contrast, infected cells (at 48–72 h post-infection (p.i.)) became resistant to apoptosis (Fig. 1, b and c). Microscopic analysis of anti-p73α-labeled U373MG showed a nuclear staining in cisplatin-treated cells, contrary to the untreated ones (Fig. 1d). This reflected an expression of p73β that could be correlated with acquisition of cisplatin sensitivity, as demonstrated previously in HCT116–3 cells (21). HCMV-mediated resistance to cisplatin was not due to the absence of p73 expression since infected cells were still labeled with anti-p73 (Fig. 1d). Furthermore, HCMV alone did not sensitize cells to apoptosis (Fig. 1, a–c), albeit infected cells expressed p73β (Fig. 1d).
FIG. 5. HCMV infection increased expression of ΔN-p73α protein in IMR-32 cells and inhibited p53 transcriptional activity and apoptosis. IMR-32 cells were either mock-infected (n.i.) or infected for the indicated times with HCMV (m.o.i. of 3). Cells lysates were submitted to SDS-PAGE and blotted with monoclonal antibody directed against viral immediate-early proteins (IE1 and IE2) or anti-p73 antibodies (b). Mock-infected (n.i.) or infected cells (6 or 24 h) were treated (+) or not treated (−) with actinomycin D (Act D) at 2 µM for an additional 15 h p.i. Expression of p21 in cell extracts was analyzed by SDS-PAGE and Western blotting using a mouse anti-p21 monoclonal antibody.

Moreover, the truncated protein comigrated with the product of a ΔN-p73α adenovirus containing a cDNA of p73α from using an alternate promoter (Fig. 3a) as described (17, 23), and U373MG cells neither expressed transcripts lacking exon 2 constitutively (16) nor following infection by HCMV (data not shown). It is noticeable that HCMV induced steady state levels of p73α, and surprisingly, that the level of ΔN-p73α expression dramatically increased during the course of infection. To determine whether overexpression of ΔN-p73α reflected an increase in gene transcription as compared with that of p73α, reverse transcription-PCR analyses were performed with specific primers. Fig. 3b shows that mRNAs encoding both truncated and full-length p73α were expressed at similar levels and at the same ratio throughout infection with HCMV, suggesting that stabilization of the ΔN-p73α protein rather than increased transcription of the gene could account for its overexpression. Accordingly, we deduce that anti-p73 labeling in HCMV-infected cells in Fig. 1d mainly reflected the expression of the ΔN-p73α isoform.

ΔN-p73α Exerts a Dominant Negative Effect on Both p73α- and p53-dependent Apoptosis—To determine whether human ΔN-p73α could exhibit an inhibitory activity on cisplatin-induced apoptosis in U373MG and SK-N-AS, cells were infected with recombinant adenoviruses expressing p73α or ΔN-p73α. Two days after infection, cells were exposed to cisplatin for 48 h, and apoptosis was determined by Western blotting of PARP cleavage. Both cell lines were sensitive to cisplatin following infection with a control adenovirus (Fig. 4, Ad-β-galactosidase) even at a higher extent in SK-N-AS than in U373MG cells, as observed above in Fig. 2. Control adenovirus alone had no effect on cell survival, but ectopic expression of p73α or ΔN-p73α induced apoptosis in both cell lines (Fig. 4, Ad-p73α). Upon combination of Ad-p73α infection and cisplatin treatment, no cell survival was observed, suggesting that under the overproduction of p73α, completion of apoptotic activity required an additional signal. Infection with Ad-ΔN-p73α rescued both cell lines from cisplatin-induced cell death, demonstrating that the truncated isoform exerted a dominant negative activity on both p73- and p53-dependent apoptosis.

To demonstrate that HCMV-induced stabilization of ΔN-p73α was responsible for the resistance of infected cells to...
p53-dependent apoptosis, a neuroblastoma cell line IMR-32 that expresses both functional p73 and p53 proteins was used. Previous studies demonstrated that following exposure of IMR-32 to actinomycin D (ActD) at concentrations that induce DNA damage, p73 was not activated contrary to p53 (17). To assess the effect of HCMV infection on apoptosis of IMR-32, cells were infected with HCMV and treated with ActD. Ensured that IMR-32 supported HCMV infection (Fig. 5c) and that most cells were infected, p73 protein expression was assayed by Western blotting. Fig. 5b shows that infection with HCMV induced an overexpression of ΔN-p73α in IMR-32 that started early (at 4 h p.i.) after virus inoculation. Then, sensitivity to apoptosis was analyzed by Western blotting of PARP cleavage. As expected, no cleavage of PARP was observed in uninfected and untreated cells. In cells that have been treated with ActD, full-length PARP was no longer recovered, reflecting apoptosis of all the cells. Following infection by HCMV, apoptosis was observed as revealed by the appearance of the 83-kDa cleavage product of PARP, which could reflect the cytotoxic effect of the virus on IMR-32. In contrast, when cells were infected with HCMV prior to treatment with ActD, full-length PARP was recovered at a similar extent to that observed in infected cells, suggesting that HCMV infection rendered IMR-32 cells resistant to ActD-induced apoptosis. Since in IMR-32 actinomycin D triggers p53 transcriptional activity (17), we asked whether HCMV infection could disrupt activation of the p53-responsive gene p21. As expected, exposure of uninfected cells to ActD induced a high level of p21 protein expression (Fig. 5d). Conversely, ActD treatment failed to induce p21 expression in HCMV-infected cells, suggesting that overexpression of ΔN-p73α could be responsible for the inhibition of p53-dependent transactivation (Fig. 5d). Collectively, our results demonstrate that HCMV infection blocked p73 and p53 apoptosis in infected cells through overexpression of ΔN-p73α.

**DISCUSSION**

Cell death is an important mechanism that determines the size and shape of the vertebrate nervous system. Converging evidences stress that neuronal death is a p53-dependent process and that multiple factors modulate cellular competence to die (24). Many viruses are capable of inhibiting p53 either through direct interactions between viral proteins and p53 or by interfering with cellular controls of p53 activity. As a result, an abnormal cell survival and a viral persistence are observed (25). HCMV is postulated to have developed various strategies to block apoptosis (14, 15, 25). However, recent studies suggested a yet uncovered mechanism for HCMV-mediated anti-apoptotic activity that may involve a cellular up-regulation of ΔNp73, a negative regulator of p53 (17). p73-deficient sympathetic neuroblasts are highly susceptible to p53-mediated apoptosis (18). In contrast, HCMV-infected neuroblasts are almost totally resistant to stress-induced apoptosis (4), raising the assumption that HCMV-induced resistance to apoptosis might be a p73-dependent process.

To address this question, various human cell lines expressing either functional p53 or p73α or both were used, and p73 expression status and resistance to stress-induced apoptosis before and after HCMV infection were evaluated in these cells. It was found that (i) only p73-expressing cells developed resistance to apoptosis after infection, (ii) resistance to cell death was associated with an accumulation of ΔN-p73α isoforms, and (iii) ectopic expression of ΔN-p73α isoforms in p73-deficient cells is per se sufficient to confer resistance to apoptosis. Finally, we demonstrated that ΔN-p73α accumulation resulted from protein stabilization rather than in an increased level of synthesis. The first cellular event observed after cell infection is the disruption of promyelocytic leukemia nuclear bodies (26), structures implicated in the proteasomal-mediated degradation of ubiquitinated proteins (27). Thus, various proteins have been shown to accumulate in HCMV-infected cells (5, 8), p73α and ΔN-p73α isoforms are both sensitive to proteasome degradation (28, 29). Interestingly, in HCMV-infected cells, ΔN-p73α was found to accumulate much more than p73α, which could be explained by the distinct cellular distribution of the two isoforms (29). Finally, it can be concluded that HCMV infection induces resistance to apoptosis through an up-regulation of ΔN-p73α. It is noteworthy that ectopically expressed ΔN-p73α is per se sufficient for p73-deficient cells to become resistant to p53-dependent apoptosis. The fact that ΔN-p73α expression is required prior to infection to ensure resistance to cell death identifies ΔN-p73α as a host factor involved in viral latency and persistence. Importantly, in mouse brain, ΔN-p73α transcripts have been reported to be the predominant TP73 products (17) and thus, ΔN-p73α is the major isoform expressed (17, 18).

Expression of ΔN-p73α transcripts is controlled by a P3 promoter located into the large intron 3 of TP73 (17). A recent study has suggested that in vitro, P3 may be regulated by p53 (23). In fact, strong evidences point out p73 itself as the factor controlling P3 activity in vivo: (i) distributions of p73α and ΔN-p73α transcripts are strictly overlapping in brain, although their relative concentration is variable (17); (ii) p53-deficient mice normally express p73α and ΔN-p73α transcripts; and (iii) in vitro, p73 can also efficiently transactivate P3 promoter (data not shown). It is therefore suggested here that one of the main functions of TP73 could be the production of a dominant negative p53 activity, ensuring neuronal survival. Intriguingly, since ΔN-p73α also inhibits p73 transactivation (17), it is likely that the regulation of ΔN-p73α activity is mainly a post-transcriptional process, supposedly controlling protein stability. It can be speculated that any interference with ΔN-p73α regulation will have implications in development and oncogenesis. For instance, virus-mediated ΔN-p73α accumulation can be predicted to immortalize Cajal-Retzius cells and to induce abnormal cortical migration with subsequent lissencephaly and/or epilepsy-like syndromes. In developing mice, indeed, mCMV was found to alter cortical neuronal migration (30), and in humans, epilepsy and disorders of cortical development in children with HCMV congenital infection have been described (31). On the other hand, by the induction of drug resistance and alteration of programmed cell death, cytomegalovirus infection may be implicated in the etiology and/or progression of malignancies such as neuroblastoma (4, 32). More generally, we hypothesize that all the viruses of the β-herpes family known to induce the inhibition of apoptosis, such as herpes simplex virus type 1, Epstein-Barr virus, and CMV, share the same capacity of up-regulating either ΔN-p73α or the other homologous protein ΔN-p63α (33) and that these viral infections are one of the major causes of drug resistance in cancer.

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