Levels of p53 in Epstein-Barr Virus-Infected Cells Determine Cell Fate: Apoptosis, Cell Cycle Arrest at the G1/S Boundary without Apoptosis, Cell Cycle Arrest at the G2/M Boundary without Apoptosis, or Unrestricted Proliferation

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The marked increases in p53 and p21/WAF1 levels that occur during Epstein-Barr virus (EBV) infection and the generation of immortal B lymphoblastoid cell lines (LCL) do not lead to growth arrest or apoptosis, although increasing wild-type (wt) p53 levels in EBV-infected cells by transfection or DNA damage induce these effects. We hypothesized that the concentration of p53 relative to that of LMP1 determines whether EBV-infected B cells undergo growth arrest and apoptosis. Cell cycle arrest and apoptosis were evaluated in LCL expressing varying p53 levels achieved by treating the cells with increasing concentrations of cisplatin, and we supplemented this approach with experiments in EBV-infected Burkitt’s lymphoma (BL) cells transfected with a temperature-sensitive (ts) mutant human p53 and studies in LCL infected with recombinant adenoviruses expressing wt and ts mutant p53. Small increases in p53 and p21/WAF1 led to cell cycle arrest at the G2/M boundary, but not to apoptosis; moderate increases resulted in growth arrest at the G1/S boundary, also without apoptosis; and large increases also induced apoptosis. These results confirm the hypothesis and reveal unanticipated complexities in cell cycle regulation by p53.

INTRODUCTION

The protein encoded by the wild-type (wt) p53 gene possesses potent tumor suppressor functions in vivo. Evidence for this conclusion comes from the demonstration that missense mutations leading to inactivation of the p53 protein, or loss of the gene, represent the most common genetic abnormality in human malignancies because they characterize >50% of a large variety of tumor types (Greenblatt et al., 1994; Hollstein et al., 1991, 1994). Furthermore, mice lacking p53, or expressing mutant p53, exhibit a markedly increased incidence of tumors (Donehower et al., 1992; Lavigueur et al., 1989; Williams et al., 1994). In addition, several DNA tumor viruses, including SV40, adenovirus, and human papillomavirus, encode proteins that interact with and inactivate the nuclear protein encoded by the p53 gene (Lechner et al., 1992; Ludlow, 1993; Sarnow et al., 1982). The mechanisms responsible for the tumor suppressor functions of the p53 gene represent a focus of intense study. In this regard, in vitro studies have documented a number of functions for p53 in various cell types, including regulation of cell cycle progression at the G1/S boundary (Ginsberg et al., 1991; Hunter, 1993), induction of apoptosis (Yonisch-Rouach et al., 1991), transcriptional activation of various genes involved in cell cycle regulation (El-Deiry et al., 1993; Harper et al., 1993; Hunter, 1993; Marx, 1994), and triggering of cellular differentiation (Aloni-Grinstein et al., 1993; Shaulsky et al., 1991).

We are interested in the mechanisms that enable Epstein-Barr virus (EBV), a DNA tumor virus of the γ-herpesvirus family, to infect and transform human B cells and thereby generate permanently dividing immortal lymphoblastoid cell lines (LCL) that express a subset of 11 EBV genes, termed latent genes. B cell immortalization requires that EBV permanently dysregulate the cellular mechanisms that regulate cell cycle progression and apoptosis, processes that are p53 dependent in a number of cell types.

In earlier studies, we observed that de novo infection of normal B cells with EBV rapidly increased basal p53 levels by ~10-fold (Chen and Cooper, 1996); other investigators have reported similar findings (Allday et al., 1995b; Szekely et al., 1995). The increase in p53 levels was shown to be mediated by the EBV latent cycle protein, LMP1, via activation of the nuclear factor (NF)-κB transcription factor (Chen and Cooper, 1996); LMP1, a membrane protein, possesses oncogenic properties in certain cell types (Wang et al., 1985). The p53 induced by EBV infection was wt because it was transcriptionally active (Allday et al., 1995b; Chen and Cooper, 1996), a finding that indicates that EBV does not encode a p53-complexing or -inactivating protein, which is in contrast to the other DNA tumor viruses noted above.

It is certainly surprising that EBV increases p53 levels...
during the process of immortalizing B lymphocytes because overexpression of wt p53 in a number of cell types blocks cell cycle progression at the G1/S boundary and leads to apoptosis (Lane, 1992; Levine, 1997; Oren, 1992). This also applies to lymphocytes because expression of wt p53 in EBV-negative Burkitt's lymphoma (BL) cell lines bearing endogenous nonfunctional, mutant p53 resulted in cell cycle arrest at the G1/S boundary and apoptosis (Okan et al., 1995; Ramqvist et al., 1993). The induction of increased levels of p53 in B cells by EBV early in infection requires that the virus also possesses mechanisms to counteract the potentially lethal properties of the increased concentrations of p53. The EBV membrane protein LMP1 fulfills this role because it upregulates intracellular levels of bcl-2 and A20, two antiapoptotic proteins (Henderson et al., 1991; Fries et al., 1996; Laherty et al., 1992). LMP1 also blocks apoptosis triggered by overexpression of wt p53 in EBV-negative BL cells bearing mutant p53 (Okan et al., 1995). These actions of LMP1 could clearly be responsible for preventing p53-induced apoptosis during the process of immortalization, as well as indefinitely, because LCL also manifest increased p53 levels and express LMP1.

In apparent conflict, however, overexpression of wt p53 in LCL by transfection blocked cell cycle progression at the G1/S boundary and induced apoptosis (Allday et al., 1995a, 1995b). LMP1, which is present in substantial concentrations in LCL, was clearly unable to block apoptosis triggered by transfection with wt p53. Increasing wt p53 concentrations in LCL by treatment of the cells with cisplatin, a genotoxic agent, yielded the same results (Allday et al., 1995a, 1995b).

These discrepant findings in EBV-infected cells can be reconciled if the concentration of p53 relative to that of LMP1 in EBV-infected cells determines whether the cells proceed through the cell cycle and continue to divide or, alternatively, respond with cell cycle arrest and/or apoptosis. The present studies were initiated to evaluate this hypothesis by further exploring the relationship among p53 expression, growth arrest, and apoptosis in EBV-infected cells. Two primary approaches were used. First, a human p53 temperature-sensitive (ts) mutant (Val143Ala), which exhibits a mutant nonfunctional conformation at 37°C and a transcriptionally active wt conformation at 32°C (Baker et al., 1990; Zhang et al., 1994), was expressed in LCL and in an EBV-negative BL cell line before and after infection with EBV. Second, varying levels of wt p53 were systematically induced in LCL by treatment of the cells with increasing concentrations of cisplatin. The cells were all evaluated for p53 and p21/WAF1 levels and for cell cycle arrest and apoptosis. The findings collectively show that p53 triggered growth arrest and apoptosis is dose dependent in EBV-positive B cells. They further indicate that small increases in cellular p53 and p21/WAF1 lead to cell cycle arrest at the G2/M boundary but not to apoptosis; moderate in-creases mediate cell cycle arrest at the G1/S boundary, also without apoptosis, whereas large increases also induce apoptosis. Finally, they demonstrate that although EBV proteins expressed in type III latency inhibited p53-mediated apoptosis, they lacked the ability to block p53-induced cell cycle arrest at the G1/S or G2/M boundaries.

RESULTS

EBV proteins block p53-induced apoptosis in BL and LCL

BL41, an EBV-negative cell line that endogenously expresses high levels of mutant p53 (Farrell et al., 1991), was stably transfected with a human p53 ts mutant (Val143Ala) (Baker et al., 1990; Zhang et al., 1994). This p53 ts mutant has a mutant conformation at 37°C and a wt conformation at 32°C (Zhang et al., 1994). Western blotting analyses revealed a single p53 band at 37°C and two bands at 32°C (Fig. 1); the faster band was absent in untransfected cells and in cells transfected with vector alone and thus likely represents wt p53, with the faster mobility being due to phosphorylation differences, as has been described previously (Ullrich et al., 1992). The mobility difference is not due to polymorphism at codon 72 because endogenous p53 and the ts mutant both express arginine at codon 72 (Baker et al., 1990; Farrell et al., 1991). At 37°C, BL41 cells expressing the p53 ts mutant, the parent cells, and the cells transfected with empty vector exhibited identical growth characteristics (Fig. 2). At 32°C, the p53 ts mutant-bearing cells died rapidly, with ~50% cell loss occurring 30 h after placement of the cells at 32°C (Fig. 2). These data are in agreement with previous studies with BL41 cells transfected with a murine p53 ts mutant (Ala135Val) (Ramqvist
et al., 1993). Cell death was due to apoptosis, as determined morphologically, and by DNA fragmentation (not shown), as previously shown (Ramqvist et al., 1993).

Next, we evaluated the effect of EBV infection on p53-induced apoptosis in BL41 cells. BL41 cells can be stably infected with EBV (Calender et al., 1987; Wang et al., 1990) despite expression of very low levels of the EBV receptor CD21. EBV infection of BL41 cells yielded type III EBV infection, with EBNA2 and LMP1 expression levels comparable to those found in LCL (Fig. 3A). Bcl-2 levels were also upregulated in EBV-infected BL41 cells (Fig. 3B). EBV infection of the BL41 cells prevented apoptosis induced by the human p53 ts mutant at the permissive temperature (Fig. 2), likely via the actions of LMP1, which has been reported to block p53-mediated apoptosis via bcl-2 induction (Okan et al., 1995).

The effects of p53 overexpression in LCL were also evaluated. However, because LCL are difficult to transfect, p53 overexpression was induced by infection with a recombinant adenovirus containing the human ts p53 mutant. Western blotting analyses showed a 3.5-fold increase in p53 levels, manifested as an increased density of a single p53 band at either 37°C or 32°C (Fig. 4A). LCL expressing the human p53 ts mutant were resistant to apoptosis at either temperature 3 days after adenovirus infection (Fig. 4B). In other studies, significant apoptosis did not occur over a 7-day period of observation (not shown). Because of concern about recombination between transfected mutant p53 and endogenous wt p53 in the cells at the permissive temperature, with the assumption of a dominant negative phenotype (Milner and Medcalf, 1991), LCL were also infected with a recombinant adenovirus containing wt p53. This yielded a 4-fold increase in p53 levels at both temperatures (Fig. 4A), and as observed with the p53 ts mutant, the cells were not susceptible to apoptosis at either temperature (Fig. 4B).

The lack of apoptosis in these studies contrasts with the report that wt p53 overexpression in LCL induces apoptosis (Allday et al., 1995b). It is unlikely that the method used to achieve overexpression of p53 in LCL in the current studies (i.e., infection with a recombinant virus compared with transfection with a plasmid containing an inducible promoter) accounts for the different results obtained in this and the previous study (Allday et al., 1995b). We postulated that a critical relationship between p53 and LMP1 concentrations determines whether apoptosis occurs and directly addressed this hypothesis in the cisplatin experiments described below.
EBV proteins do not block p53-induced upregulation of p21/WAF1 and cell cycle arrest at the G1/S boundary in BL and LCL.

In this study, the effect was evaluated of EBV infection of BL41 cells on p53-induced induction of p21/WAF1 and growth arrest. Augmented p21/WAF1 levels were observed in the transfected BL41 cells at the permissive temperature, and this induction was not altered by EBV infection (Fig. 5A). Cell cycle analyses carried out 24 h after placement of the cells at 32°C showed that the p53 ts mutant bearing BL41 cells was arrested at the G1/S boundary because 71.5% of the cells were in G1 and 27.1% were in the other phases of the cell cycle, which is in contrast to 55.8% in G1 and 42.1% in S and G2/M together in the same cells at 37°C (Fig. 5B).

EBV infection had no effect on cell cycle arrest at the G1/S boundary in BL41 cells bearing the p53 ts mutant because 71.5% of the uninfected cells were in G1 and 271% were in the other phases of the cell cycle, whereas 72.7% of the EBV-infected BL41 ts p53 mutant-bearing cells were in G1 and 25.5% were in S plus G2/M together at 32°C. Therefore, none of the EBV genes expressed in type III latency possess the ability to block p53-induced p21/WAF1 expression and cell cycle arrest at the G1/S boundary. A previous study had shown that LMP1 lacks this property (Okan et al., 1995), but other EBV proteins were not assessed. Studies in LCL yielded data consistent with this interpretation because p21/WAF1 was induced only at 32°C in LCL infected with adenovirus containing the human p53 ts mutant, where the ts mutant exhibits a wt conformation, but the cell cycle regulatory protein was induced at 32°C, as well as at 37°C, in LCL infected with adenovirus encoding wt p53 (Fig. 6A). Cell cycle arrest occurred at the G1/S boundary in LCL infected with adenovirus containing ts mutant p53 at 32°C (69.1% in G1 and 29.4% in the other phases of the cell cycle vs 48.9% in G1 and 47.9% in S and G2/M together at 37°C). This was also true of LCL infected with virus-expressing wt p53 at both 32°C (68.9% in G1 and 29.4% in S plus G2/M) and 37°C (63.5% in G1 and 35.9% in the other phases of the cell cycle) (Fig. 6B).
The effects of p53 on apoptosis and cell cycle arrest are dose dependent

The comparative studies in BL and LCL overexpressing p53 described above suggest that p53-mediated apoptosis and cell cycle arrest are dependent on a critical relationship between the concentrations of p53 and LMP1 in the cells. We directly evaluated this hypothesis by examining p53-induced apoptosis, p21/WAF1 induction, and cell cycle arrest in cells expressing a range of concentrations of wt p53. Because of the technical difficulties inherent in achieving varying expression levels of transfected genes in cells, we evaluated the possibility that treatment of LCL with increasing cisplatin concentrations would yield cells with incremental increases in endogenous wt p53 levels. This proved to be the case because treatment of LCL with cisplatin concentrations between 0.16 and 20 μg/ml for 18 h yielded LCL with increasing p53 concentrations that ranged from 1.2-fold to 12-fold over endogenous levels (Table 1, Fig. 7). LMP1 levels in the cells were only modestly reduced by treatment with cisplatin (0.4-fold decrease at 20 μg/ml cisplatin) (Fig. 8). Interestingly, the LCL were resistant to apoptosis when treated with cisplatin concentrations of 5.0 μg/ml, which were associated with p53 levels up to 15.6-fold above the endogenous concentration (Table 1). Treatment with 5 μg/ml cisplatin produced an ~10-fold increase in the p53 concentration and resulted in 30% apoptosis, whereas 10 μg/ml cisplatin yielded an ~15.6-fold increase in p53 levels and 55% apoptosis (Table 1).

**TABLE 1**

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<tr>
<th>Consequences of Treatment of JR LCL with Cisplatin</th>
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<tr>
<td>Cisplatin concentration (μg/ml)</td>
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<td>p53 induction (approximate)</td>
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Apoptosis was verified by morphological examination, by demonstrating DNA fragmentation on gels, and by cell cycle analysis (see below). The slight apparent decrease in p53 levels at the highest cisplatin concentration likely reflects the reduced numbers of cells present and possibly also degradation.

Induction of p21/WAF1 in the cisplatin-treated LCL lagged behind the p53 induction curve at cisplatin concentrations of 1.25 μg/ml but exactly mimicked the levels of p53 induction when this reached ~5-fold over endogenous levels (Table 1, Fig. 7). Cell cycle analyses of the cells, however, revealed several unanticipated findings (Table 1, Fig. 9). First, cells treated with the lowest cisplatin concentrations (0.16±0.63 μg/ml), which yielded ~12- to 2.5-fold increases in p53 concentrations but little increase in p21/WAF1 levels (maximum of 1.4-fold), were growth arrested at the G2/M boundary (Table 1, Fig. 9). This is clearly shown by the progressive decrease in cells in G0/G1 and the progressive increase in cells in S plus G2/M. These cells are not undergoing apoptosis because subdiploid, apoptotic fragments are absent in these samples in the cell cycle analyses (Fig. 9). Second, LCL treated with intermediate cisplatin concentrations (125±2.5 μg/ml), which yielded moderate increases in p53 levels (~3.2- to 5.4-fold), and comparable increases in p21/WAF1 levels, were growth arrested at the G1/S boundary, as indicated by the increase in cells in G0/G1 and the decrease in the proportion of cells in S and especially G2/M (Table 1, Fig. 9). The cell cycle analyses revealed insignificant levels of apoptosis in these samples, as shown by the virtual absence of cells with a DNA content of less than the diploid G0/G1 peak (Fig. 9). Finally, LCL treated with ~5.0 μg/ml cisplatin concentrations, which exhibited ~10-fold increases in p53 and p21/WAF1, were not only growth arrested at the G1/S boundary but also actively undergoing apoptosis, as indicated by cell morphology and the increase in subdiploid fragments in the cell cycle studies (Table 1, Fig. 9). Apoptosis in LCL treated with 5, 10, and 20 μg/ml cisplatin was confirmed by the use of gel electrophoresis to demonstrate DNA fragmentation (Fig. 10).

**DISCUSSION**

Unlike other DNA tumor viruses, including SV40, adenovirus, and human papillomavirus, EBV gene products do not directly inactivate the critical p53 regulator of cell cycle progression and apoptosis. Rather, EBV, via the actions of LMP1, prevents apoptosis triggered by elevated levels of wt p53 (Okan et al., 1995). These actions of LMP1 are probably mediated by bcl-2 or A20, or both, two ant apoptotic proteins that are induced by the viral latent protein (Henderson et al., 1991; Laherty et al., 1992; Okan et al., 1995). LMP1, however, does not prevent p53-induced cell cycle arrest at the G1/S boundary (Okan et al., 1995). Thus EBV, via LMP1, interferes with certain p53 functions but via different mechanisms than the other DNA tumor viruses noted above. Curiously, LMP1 is also responsible for the induction of the increased levels of transcriptionally active p53 that occur early during EBV infection and persist thereafter in EBV-transformed cells (Allday et al., 1995b; Chen and Cooper, 1996).
The present studies were initiated to determine why the increased levels of p53 that characterize EBV infection do not induce either apoptosis or cell cycle arrest, especially because overexpression of wt p53 in LCL achieved by transfection or cisplatin treatment and in BL by transfection led to growth arrest at the G1/S boundary and to apoptosis (Allday et al., 1995a, 1995b; Okan et al., 1995). Although LMP1 could have been responsible for abrogating the potential apoptotic actions of increased p53 levels produced by EBV infection in our previous studies (Chen and Cooper, 1996), because it has this property after overexpression in BL as noted above (Okan et al., 1995), the LMP1 levels prevailing in LCL were unable to block apoptosis triggered by increased p53 levels induced by transfection or cisplatin treatment (Allday et al., 1995a, 1995b).

We hypothesized that a critical relationship between the levels of p53 and LMP1 determines whether EBV-infected B cells respond to increased p53 levels with cell cycle arrest and apoptosis to explain these various discrepant findings. To evaluate this hypothesis, we examined cell cycle arrest and apoptosis in LCL expressing increasing concentrations of p53 achieved by treating the cells with increasing concentrations of the genotoxic agent cisplatin; LMP1 levels were not significantly altered by cisplatin. Apoptosis induced by DNA damage is p53 dependent (Clarke et al., 1993; Kastan et al., 1991; Lowe et al., 1993). These experiments were supplemented with studies in EBV-infected BL41 cells transfected with ts mutant human p53 and with experiments in LCL infected with recombinant adenoviruses expressing either wt or the ts mutant p53. The results obtained here not only confirm the hypothesis and thereby reconcile the discrepant results obtained in the published studies cited earlier but also reveal unanticipated complexities in cell cycle regulation by p53.

A 10-fold increase in p53 and p21/WAF1 concentrations over endogenous levels in LCL resulted in cell cycle arrest at the G1/S boundary and apoptosis. These findings are consistent with published studies in LCL induced to overexpress high levels of wt p53 by either transfection or treatment with 10 μg/ml cisplatin (Allday et al., 1995a, 1995b). Such p53 and p21/WAF1 levels are 100-fold over those prevailing in uninfected human B cells because EBV infection itself increases p53 100-fold and triggers an even greater elevation in p21/WAF1 levels (Chen and Cooper, 1996). These levels of p53 apparently exceed those that can be regulated by the levels of LMP1 in the cells. A lesser increase in p53 and p21/WAF1 levels (2.5- to 5-fold) also triggered cell cycle arrest at the G1/S boundary but did not lead to apoptosis. The LMP1 levels in these cells were apparently sufficient to block p53-induced apoptosis. The studies in LCL infected with recombinant adenovirus expressing either ts mutant or wt p53 are entirely in accord with these findings because the 3.5- to 4-fold increase in p53 levels produced cell cycle arrest at the G1/S boundary without apoptosis. This is also true of the experiments in EBV-infected BL41, where an estimated 3- to 4-fold increase in wt p53 concentrations (compared with LCL levels) resulted in G1/S cell cycle arrest without apoptosis.

Entirely unanticipated, however, was the finding that small ~12- to 2.5-fold increases in p53 resulted in cell cycle arrest at the G2/M boundary, again without apoptosis. Because these slight increases in p53 were not accompanied by significant increases in p21/WAF1, it is probable that cell cycle arrest at this checkpoint is independent of the actions of this cell cycle regulatory protein. Some evidence has previously been obtained for p53-induced cell cycle arrest at the G2/M boundary in fibroblasts (Stewart et al., 1995), but such findings have not been reported in B cells. The mechanism by which p53 induces cell cycle arrest at the G2/M boundary may be dependent on p53-mediated induction of the 14-3-3σ proteins (Hermeking et al., 1998).

A second finding derived from these studies is that infection of BL41 with EBV prevents p53-induced apoptosis but not growth arrest at the G1/S boundary. Similarly, LCL are growth arrested at either the G1/S or the G2/M boundaries, depending on the p53 concentration. These data indicate that none of the nine EBV latent proteins expressed in EBV-infected BL41 cells or in LCL, both of which exhibit type III latency, possess the ability to prevent growth arrest at the G1/S or G2/M cell cycle checkpoints induced by modest-to-moderate increases in levels of cellular wt p53 over those found in LCL. LCL, however, do not exhibit growth arrest. Therefore, the 10-fold increase in p53 levels that LCL exhibit relative to uninfected B cells must not be sufficiently high to induce growth arrest of the cells. Alternatively, EBV gene products may possess some ability to interfere with the G2/M growth-arresting properties of modestly increased p53 levels, such as those prevailing in LCL. These aspects are under investigation.

The most important conclusion emerging from these studies is that p53-dependent effects on cell cycle regulation and apoptosis represent a continuum, with influences on cell cycle progression being evident with even very small increases in p53 levels and with entirely different effects being evident at low, intermediate, and high p53 levels. Although these actions are clearly unrelated with the growth-promoting effects of EBV latent cycle proteins, they show that p53 actions are not all or none but rather are dose dependent. They also are not fundamentally different for EBV-infected and uninfected B cells because the induction of proliferation of normal EBV-uninfected cells by treatment with anti-CD40 plus interleukin-4 increased cellular p53 levels to the same extent as EBV infection (Allday et al., 1995b). Ligation of the B cell antigen receptor (BCR) on EBV-uninfected B cells also led to rapid increases in p53 and p21 (Wu et al., 1998). In the latter study, transfection approaches
showed that p53 and p21 were directly involved in the signaling pathway leading to apoptosis after BCR ligation. The concentrations of p53 that produce the various described effects are likely to differ for various cell types, including B cells, not only because of the different levels of LMP1 expression in various LCL but also as a result of other unknown cell-specific effects on proliferation and growth regulation.

MATERIALS AND METHODS

Cells

BL41 is an EBV-negative BL cell line that bears mutant, nonfunctional p53 (Farrell et al., 1991). EBV-infected BL41 was generated by incubation with EBV of the B95-8 strain, prepared as described previously (Nemerow and Cooper, 1981). The LCL used in this study (JR) was also generated by infection with B95-8 strain EBV. The cells were maintained in RPMI 1640 medium with 10% fetal calf serum.

Plasmids, transfections, recombinant adenovirus preparations, and infection

Expression vectors containing the human ts p53 mutant Val143Ala (pC53-SCX3) and wt human p53 (pC53-SN3) were obtained from Bert Vogelstein (Baker et al., 1990; Chen et al., 1992). BL41 cells were transfected by electroporation (1 × 10^7 cells, 250 V, 960 μF, in a Gene Pulser from BioRad, Richmond, CA). After culture for 3 days, the cells were selected with G418 at a concentration of 2 mg/ml for ≥2 weeks.

A recombinant adenovirus shuttle vector (pAd/RSV) was generated by subcloning the RSV promoter-BGH poly(A)^+ expression cassette from the pRc/RSV vector (InVitrogen, Carlsbad, CA) into the pE1sp1B (Microbix Biosystems, Toronto, Canada) vector using BamHI and BgIII sites. The wt and mutant p53 cDNAs were inserted into the HindIII–XbaI site of pAd/RSV. Then, 15 μg of each construct and an equivalent amount of the adenovirus packaging plasmid pJM17 (Microbix-Biosystems) were cotransfected into the E1 transcomplementing cell line 293 using calcium phosphate. The recombinant adenoviruses, generated by homologous recombination, were isolated by plaque formation on 293 cells as previously reported (Huang et al., 1997). High-titered stocks (1 ± 2 × 10^12 virions/ml) of the recombinant adenoviruses were grown in 293 cells and purified by density gradient ultracentrifugation. A control recombinant adenovirus lacking a cDNA insert was constructed through the use of pAd/RSV and pJM17. LCL were infected with 10^4 viral particles per cell for 24 h, after which the cells were incubated at 32°C for an additional 48 h. Control β-Gal recombinant virus was used for the determination of transfection efficiency. More than 90% of the cells were infected by the recombinant viruses.

Growth assays

Cells were seeded at a density of 1 × 10^6/ml and cultured at 37°C or 32°C. The cells were counted daily, and viability was determined by trypan blue exclusion or by the MTT assay (Chemicon, Temecula, CA).

Induction of varying levels of p53 and p21 in LCL

LCL were treated with varying concentrations of cisplatin (Sigma Chemical, St. Louis, MO), obtained by the addition of varying amounts of a 15 mg/ml stock solution in PBS, to incrementally increase p53 and p21 levels.

Immunoblotting assays

These were performed as described (Chen and Cooper, 1996) using 5 × 10^5 cells per lane. The DO-1 anti-p53 monoclonal antibody (mAb) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-p21/WAF1 and the bcl-2 mAbs were purchased from Oncogene Research Products (Cambridge, MA). The PE2 anti-EBNA2 and S12 anti-LMP1 mAbs have been previously described (Chen and Cooper, 1996; Mann et al., 1985; Young et al., 1989). The primary Abs were diluted with 5% dry milk in PBS and incubated with the blots for either 1 ± 2 h at room temperature or overnight at 4°C. Horseradish peroxidase-labeled goat anti-mouse IgG Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD) served as the second Ab, and signals were detected with the ECL system (Amersham Life Sciences, Arlington Heights, IL). After stripping (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL, pH 6.7, at 70°C for 30 min), the blots were reprobed with mAb to actin (clone C4, ICN Biochemicals, Aurora, OH).

Densitometric analyses were carried out on a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) using ImageQuant 4.2a software. Fold increases were calculated by dividing pixel density units of the various bands by the pixel density units obtained for the controls.

Apoptosis assays

Apoptosis was detected after washing the cells in PBS followed by staining with acridine orange (Sigma) and visual examination under a fluorescent microscope. DNA fragmentation was evaluated by a gel electrophoretic method adapted from a published procedure (Sorenson et al., 1990). In brief, cell pellets (1 × 10^6 cells), suspended in buffer containing 10 mg/ml ribonuclease A, 15% Ficoll 70, and bromphenol blue, are loaded into wells partially filled with 0.8% agarose containing 2% SDS and 125 mg/ml proteinase K. Cell lysis begins in the sample buffer and is completed during the initial electrophoresis (20 V for 1 h). Electrophoresis is continued (90 V) for 3 h, followed by rinsing of the gel in water, overnight incubation in 100 ml of pH 8.0 TE buffer con-
taining 20 μg/ml RNase A, and ethidium bromide staining.

Flow cytometric analyses of cell cycle progression

Cells were harvested; washed in ice-cold Tris-buffered NaCl, pH 7.5, containing 5 mM EDTA (TSE); fixed by the dropwise addition of 70% ethanol (–20°C) with constant gentle mixing; and stored overnight at 4°C before analysis. The fixed cells (2 × 10^6) were washed in TSE and resuspended in 1 ml of a propidium iodide (PI) solution composed of 0.025% PI in 0.1% sodium citrate, 0.01% RNase, and 0.1% Triton X-100 in TSE (Stewart et al., 1995). After ≥2 h at room temperature in the dark followed by filtration through 41-μm Spectramesh (Spectrum, Laguna Hills, CA), cell cycle status was determined by flow cytometry on a FACSort using CELQuest software (Becton Dickinson, San Jose, CA).

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