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Enhancement of UV-induced cytotoxicity by the adeno-associated virus replication proteins

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Abstract

Adeno-associated virus (AAV) normally requires co-infection of a helper virus to complete its life cycle. However, under conditions of cellular stress, such as treatment with carcinogens or ultraviolet (UV) light, a permissive intracellular environment is established and AAV completes its replicative cycle producing low levels of progeny virus. AAV DNA replication is dependent upon viral replication proteins, Rep78 and Rep68. The detailed mechanism by which these proteins interact with host cell factors is unknown. We have used a cell line (Neo6) that inducibly expresses the AAV Rep proteins to study their effects on cells that have undergone UV-induced DNA damage. Induction of Rep protein expression immediately after a sub-lethal dose of UV irradiation resulted in rapid cell killing. Those cells that die had chromatin condensation while cellular membranes remained intact, suggesting that concurrent Rep expression and UV damage induces an apoptosis-like response. However, we did not observe any DNA degradation. Thus we believe that the combination of Rep expression and UV irradiation induces cell death that shares some of the characteristics of apoptosis. UV irradiation and Rep expression induced an increase in the level of the CDK inhibitor, $p21^{Cip}$, and the appearance of modified forms of both $p21^{Cip}$ and Bcl-2. Alteration of normal expression of these cytostatic/apoptotic proteins provides insight into the intracellular targets of the AAV replication proteins.

Keywords: Adeno-associated virus; DNA damage; *rep* gene; Cytotoxicity

1. Introduction

Adeno-associated virus (AAV) is a human parvovirus that normally requires the assistance of a helper virus to efficiently complete its life cycle (reviewed in [1–3]). Adenovirus (Ad) is the most efficient helper for AAV although human papillomavirus [4], cytomegalovirus, vaccinia virus, Epstein-Barr virus [5–8] and herpes simplex virus [9] will also support AAV replication. The role of the helper virus in AAV replication is to enhance AAV replication and gene expression [24]. AAV replicates in the absence of helper virus infection when cultured cells have been synchronized or challenged with DNA-damaging agents [10–12].

The AAV genome encodes four replication (Rep) proteins. Either of the two larger Rep proteins Rep78 and Rep68 is sufficient for viral DNA replication. The two smaller Reps, Rep52 and Rep40, are involved in virus assembly. The AAV genome en-

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codes four replication (Rep) proteins that are essential for DNA replication and assembly of an infectious virus. Rep78 and Rep68 are translated from mRNAs that originate from a transcription promoter at map unit 5 (p5). Rep52 and Rep40 are translated from mRNAs that originate from a transcription promoter at map unit 19 (p19). Rep68 and Rep40 are translated from spliced mRNAs and lack about 100 amino acids from the carboxyl terminus of Rep78 and Rep52 respectively. Rep78 and Rep68 mediate AAV DNA replication by binding to a 16 bp element Rep binding site (RBS) found in the A-stem of the covalently closed end of the AAV origin of replication. Upon binding, either of the larger Rep proteins will make a site-specific strand-specific cut at a terminal resolution site (trs) 17 nucleotides from the RBS [13–15]. This cleavage is ATP dependent and results in covalent attachment of the Rep protein to the 5′ end of the viral DNA. Cleavage allows for unwinding of the closed end of viral DNA and completion of DNA synthesis. Rep proteins also bind and cleave the cellular RBS found in the chromosome 19 integration site suggesting that the endonuclease activity is required for viral DNA integration [15].

In addition to viral DNA replication, the Rep proteins regulate viral gene expression. Rep78 and Rep68 repress transcription from the p5 promoter in the presence and absence of Ad co-infections [16–18]. There is a RBS in the p5 promoter, which is essential for inhibition of p5 transcription [18,19]. In the presence of Ad co-infection, Rep78 and Rep68 activate transcription of the p19 and p40 promoters [20,21]. Rep-specific trans-activation of the p40 promoter requires the RBS in the terminal repeat (or p5 promoter), a CarG-like element in the p19 promoter and a Sp1 binding site in the p40 promoter [21]. Rep proteins also interact with cellular factors that play roles in the regulation of gene expression. Purified Rep78 and Rep68 have been reported to associate with the Sp1 transcription factor [21,22] and high mobility group non-histone protein 1 [23]. Rep52 and Rep40 proteins also repress p5 transcription in the absence of Ad infection [17,18].

Although AAV has never been conclusively linked to any pathological condition or disease, it can have profound effects on the replication of its helper virus and the infected cell. AAV inhibits Ad-induced transformation and Ad DNA synthesis (reviewed in [1,23]). Inhibition of Ad-induced tumorigenicity and cellular transformation has been attributed to the AAV terminal repeat sequences and AAV genes [25,26], whereas the effects on Ad replication have been attributed to the AAV replication (*rep*) gene [27]. The *rep* gene has also been shown to inhibit human herpesvirus [28], HIV [29,30], SV40 [31,32], and bovine papillomavirus replication [33].

In the absence of a helper virus co-infection, AAV integrates into the long arm of chromosome 19 (reviewed in [34]). AAV infection of cells has been shown to alter the expression of several cell cycle regulated genes [35], promote differentiation-like effects [36] and induce a late S-phase and/or G_2-phase cell cycle block [11,37]. These effects have been attributed to *rep* gene expression as well as the virion particle itself. A recent study has revealed that AAV infection of primary human cells induces a cell cycle block that is correlated with a decrease in retinoblastoma (Rb) protein phosphorylation and an increase in $p21^{Cip}$ gene expression [35]. In a helper virus-free environment, Rep expression impedes gene expression from heterologous promoters [17,26,29,38] and it has also been reported to block translation of the AAV *cap* gene mRNA [39]. Transient expression of the *rep* gene prevents cellular DNA replication; however, it is not known whether this is due to inhibition of cell cycle progression or a direct effect on DNA synthesis [32]. Two different cell lines have been established that express the *rep* gene under the control of inducible transcription promoters [40,41]. Both cell lines express Rep proteins that retain wild type functions. However, one of these cell lines arrests in S-phase upon induction of Rep expression [41].

Our laboratory is very interested in the interactions between the AAV Rep proteins and the host cell. In this report we describe some of our investigations into the combined effects of Rep expression and UV irradiation on cell lines that inducibly express the AAV Rep proteins. We have observed that concomitant Rep expression and UV light are cytotoxic and induce cell death that displays some of the characteristics of apoptosis. Immunoblot analyses reveal that Rep expression may exert its effects by altering the levels and post-translational modification of cell cycle and apoptosis-related proteins.

2. Materials and methods

2.1. Cells and antibodies

Neo5, Neo6 and Neo40 cell lines were derived from human embryonic kidney, 293, cells [41]. Briefly, the lines were obtained by co-transfecting 293 cells with a plasmid that expresses the AAV *rep* gene under the control of the heavy metal inducible, mouse metallothionein transcription promoter, and a plasmid that expresses the *neo* resistance gene. Individual clones were selected and screened for Rep protein expression. The Neo6 and Neo40 cells expressed functional Rep78 whereas the Neo5 cells did not express the protein. Thereafter the Neo5 line was used as a control for the Neo6 and Neo40 cells. The cells were grown in Eagle's minimum essential medium (MEM) containing 10% dialyzed fetal bovine serum, 1 mg of geneticin (Gibco BRL) per ml (708 μg/ml active component) and other antibiotics at 37°C in a 5.0% CO_2 atmosphere. Immunoglobulin G-fractionated anti-Rep rabbit antiserum was used in immunoblots as described [41]. Monoclonal mouse anti-p53 antibody (OP09) was purchased from Oncogene. Polyclonal rabbit anti-$p21^{Cip}$ antibody (SC-397), polyclonal rabbit anti-Bax antibody (SC-526), and monoclonal mouse anti-Bcl-2 antibody (SC-509) were purchased from Santa Cruz Biotechnology. Alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from Pierce. Alkaline phosphatase-conjugated goat anti-rat IgG was purchased from Sigma. Alkaline phosphatase-conjugated goat anti-mouse IgG was purchased from Life Technologies.

2.2. DNA synthesis assay

To investigate the effect of Neo6-produced Rep proteins on cellular DNA synthesis, [^{3}H]thymidine incorporation experiments were performed. Neo5, Neo6 and Neo40 cells were plated in 24-well dishes at a density of 10^4 cells/well. Cells in half of the wells were induced with heavy metal for 3 days. All the cells were then labeled with 10 μCi/ml of [^{3}H]thymidine for 24 h. At the end of labeling, cells were harvested and lysed by incubation in 10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1% SDS, 0.1 M NaCl, 0.2 mg/ml proteinase K at 37°C for 3 h. After two phenol-chloroform extractions, the DNA was precipitated with ice-cold 10% trichloroacetic acid (TCA), and the precipitates were collected by filtering the solution through GF/A glass fiber filters (Whatman). The filters were washed extensively with 10% TCA and 95% ethanol and radioactivity on the filters was determined by scintillation counting.

2.3. MTT assay

To determine cell viability, a rapid colorimetric assay was employed as described [42] with some modifications. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) stock solution was prepared by dissolving MTT in phosphate-buffered saline (PBS), pH 7.5, at a concentration of 5 mg/ml and filtered with 0.2 μm syringe filter for sterilization and to remove insoluble residue. Before use, the stock solution was diluted 10-fold in phenol red-free MEM (Life Technologies) and warmed to 37°C. The initial culture medium in 24-well plates was replaced by 250 μl of the MTT-containing MEM followed by a 4 h incubation of the plates at 37°C in a humidified 5% CO_2 atmosphere. The medium was removed by aspiration and 200 μl of stop solution (0.04 N HCl in isopropanol) was added to each well. After shaking vigorously at room temperature for 1 h to dissolve the formazan crystals, 100 μl of stop solution from each well were transferred to a 96-well plate and absorbance at 595 nm was measured on a Thermomax microplate reader (Molecular Device). Percentage viability was determined from the absorbance of the treated cultures divided by the absorbance of the culture at time zero in Fig. 1 or in the absence of CDDP in Fig. 2.

2.4. UV irradiation of cells

To investigate the dose response of Neo5 and Neo6 cells to ultraviolet (UV) light irradiation, Neo5 and Neo6 cells were seeded into 24-well plates. When cells reached about 40% confluence, the culture medium was removed and the cells were irradiated with 254 nm UV light in 0.5 ml of PBS containing 5 mM $MgCl_2$ (PBS/Mg) at various incident fluency rates with a Stratalinker UV Crosslinker (Stratagene). The PBS/Mg was then replaced by fresh

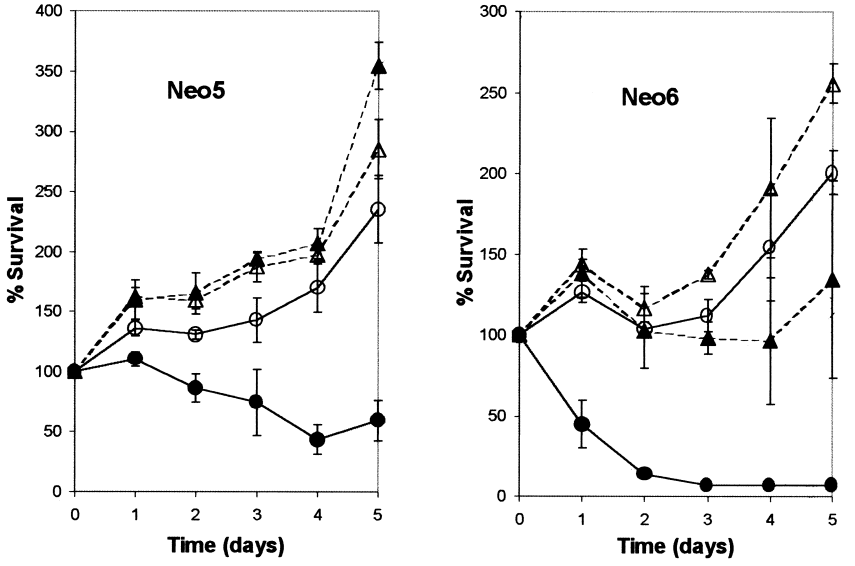

Fig. 1. UV irradiation of Rep-expressing cells. Neo5 and Neo6 cells were irradiated with a dose of 5 mJ/cm^2 at the zero time point. MTT viability assays were performed at each time point. All of the cultures were treated with UV irradiation at the zero time point. The cultures were treated with heavy metals (HM) at the following times: time zero and HM was maintained until viability assays were performed (solid lines and closed circles); 24 h before UV irradiation and then HM was removed at time zero (solid lines and open circles); 24 h before UV irradiation and HM maintained until viability assays were performed (dashed lines and closed triangles); no HM (dashed lines and open triangles). Each point is derived from an average of triplicate cultures and the experiment was repeated three times.

culture medium and the cells were incubated under normal culture conditions for 3 days. The dose response was then examined by measuring cell viability using the MTT assay. The half-lethal dose (LD_{50}) of 254 nm UV light to Neo5 and Neo6 cells was 5000 μJ/cm^2.

To study the effects of UV irradiation and Rep expression on cell viability, Neo5 and Neo6 cells in 24-well plates were irradiated in PBS/Mg with 254 nm UV light when the cells reached about 40% confluence. 100 μM $ZnCl_2$ and 2 μM $CdSO_4$ were added to cultures either 24 h before, or immediately after, UV irradiation. Cell viability was measured by MTT assays at various time points after UV irradiation.

To study the effects of *cis*-platin (*cis*-platinum(II)-diammine dichloride; CDDP; Sigma) and Rep expression on cell viability, 30–40% confluent Neo5 and Neo6 cells in 24-well plates were treated with

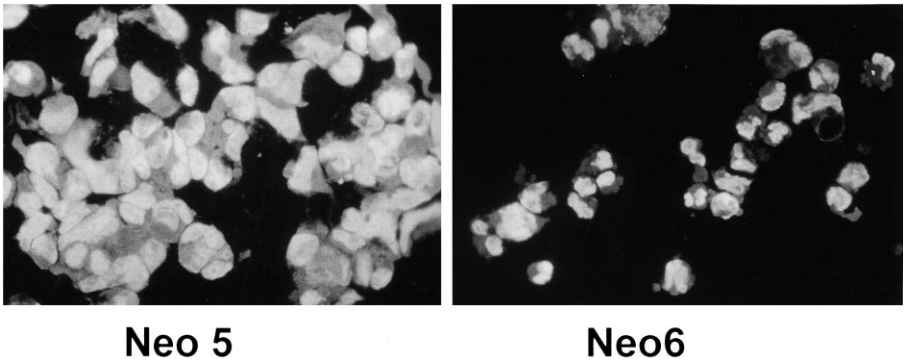

Fig. 2. Alteration of cell morphology by Rep expression and UV irradiation. Neo5 and Neo6 cells growing on coverslips were treated with heavy metal salts immediately after UV irradiation. 16 h later the cultures were fixed and stained with acridine orange to visualize nuclei. 400× magnification.

CDDP at various concentrations in MEM for 24 h at 37°C. The CDDP containing medium was then replaced by normal MEM or MEM supplemented with 100 μM $ZnCl_2$ and 2 μM $CdSO_4$. Three days later MTT assays were performed to monitor cell viability.

2.5. Protein extraction and Western analysis

For the analysis of Rep and cellular proteins, subconfluent Neo5 and Neo6 cultures were harvested 16–24 h after the various UV and heavy metal treatments. Cells were harvested by scraping into culture medium and pelleted at 1000×*g* for 5 min. The cell pellets were washed twice in PBS/Mg and incubated on ice in 100 μl of IPP buffer containing proteinase inhibitors (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 0.5% Nonidet P-40, 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 0.1 mg/ml leupeptin, 0.1 mg/ml pepstatin and 0.1 mM benzamidine). Cell debris was removed by centrifugation at 10 000×*g*, 4°C for 20 min. Protein concentrations were determined by the Bio-Rad Protein Assay according to the manufacturer's instruction. The protein samples were heated at 100°C for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, equal protein amounts were separated on 10% SDS-PAGE gels at 14 mA overnight and transferred to nitrocellulose filter membranes using a Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad) following the manufacturer's instruction. The filter was blocked in 2% dry milk in PBS for 1–2 h and then cut into strips and incubated with appropriate primary antibodies diluted 200-fold in PBS containing 1% dry milk for 2 h at room temperature or overnight at 4°C, followed by three washes, 10 min each, in PBS containing 0.05% Tween 20. Appropriate alkaline phosphatase-conjugated secondary antibodies were diluted 2000-fold in PBS containing 1% dry milk and incubated with the blots for 1 h at room temperature. After three washes with PBS-Tween 20 buffer, the blots were stained with NBT and BCIP for 5–20 min.

2.6. DNA fragmentation assay

To prepare nucleosomal DNA for use as size markers in the DNA fragmentation assay, Neo6 cells grown in 10 cm dishes were harvested by scraping into PBS/Mg buffer. After two washes in PBS/Mg, cells were lysed in 300 μl of STM-NP buffer (10 mM Tris-HCl, pH 8.0, 0.25 mM sucrose, 10 mM $MgCl_2$, 0.5% Nonidet P-40) on ice for 10 min. Cell nuclei were pelleted at 1000×*g*, 4°C for 10 min and resuspended in 300 μl of STM-NP buffer containing 5 mM $CaCl_2$. Aliquots of the nuclear suspensions were digested with 1 unit of micrococcal nuclease for 1 min or 2 min at 37°C. The reactions were stopped by the addition of 10 mM EGTA (pH 8.0) and incubated on ice for 10 min. The nuclear suspensions were diluted with protease K digestion buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% SDS) and digested with 200 μg/ml of protease K at 37°C for 24 h. DNA was extracted once in 1:1 phenol/chloroform and once in 24:1 chloroform/isoamyl alcohol and precipitated with ethanol at −70°C overnight. DNA precipitates were pelleted at 10 000×*g* for 30 min and dissolved in 20 μl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer containing 1 unit RNase A and 40 units RNase T1 (Ambion). Total DNA was isolated from Neo5 and Neo6 cells after UV and heavy metal treatment. Cells were scraped into the culture medium, pelleted and washed in PBS/Mg and DNA was isolated as described above. DNA concentrations were calculated based on the absorbance at 260 nm. 60 μg of DNA from each sample were separated on 1.5% agarose gel at 100 mA.

2.7. Cell staining

Acridine orange was used to stain Neo5 and Neo6 cells grown on coverslips after treatment using the various combinations of heavy metal and UV irradiation. 16 h after UV irradiation the cells were fixed in 95% methanol, 5% PBS at −20°C for 10 min and then stained with 10 μg/ml acridine orange (Sigma) in PBS at room temperature for 10 min. Coverslips were then mounted onto slides and examined using a Nikon Diaphot-TMD microscope with EPI-fluorescence equipment and photographed with Kodak T-MAX 400 black and white film at 400× magnification.

To test for membrane integrity, Neo5 and Neo6 cells grown on coverslips were treated with the various combinations of heavy metal salts and UV. Prior to fixation, the cells were stained with 10 μM propidium iodide (PI) in PBS at room temperature

for 5 min. After staining, the cells were washed once in PBS and mounted onto slides for examination by microscopy and photography.

3. Results

3.1. Rep expression inhibits cellular DNA synthesis

Previously, we reported on the isolation of a human 293 cell line that expresses the AAV *rep* gene under the control of the mouse metallothionein transcription promoter [41]. Expression of the Rep proteins results in inhibition of cell proliferation and an apparent S-phase cell cycle block. To determine whether the S-phase block is reflected in an inhibition of DNA synthesis, two Rep-expressing cell lines and a control line were incubated with heavy metal salts. At 72 h after induction, the cultures were radiolabeled with [^{3}H]thymidine. The cultures were harvested, DNA isolated and [^{3}H]thymidine incorporation determined by TCA precipitation and scintillation counting. Table 1 shows that the control Neo5 cell line incorporated more [^{3}H]thymidine after heavy metal induction. The higher level of tritium incorporation in the Neo6 and Neo40 cells is due to their more rapid growth rate in the absence of heavy metal induction (results not shown). However, the Rep-expressing cell lines, Neo6 and Neo40, both synthesized much less DNA after induction. These results are consistent with an S-phase cell cycle block and indicate that Rep protein expression is correlated with inhibition of cellular DNA synthesis.

3.2. UV irradiation and Rep expression are cytotoxic

AAV undergoes low level replication when cultured cells are treated with UV light and other DNA-damaging agents [10]. Helper virus-free replication is also Rep protein-dependent and cytotoxic [43]. To determine if Rep proteins confer toxicity on UV-irradiated cells, Neo5 and Neo6 cells were treated with a sub-lethal dose of UV light. The amount of UV for these experiments was determined by a standard dose-response assay in which increasing fluencies of UV were used and cell viability was evaluated 3 days later. This experiment demonstrated that there is no inherent difference between Neo5 and Neo6 cells with respect to sensitivity to UV irradiation (results not shown). To determine whether Rep expression affects cell viability after irradiation, Rep protein expression was induced either 24 h before, or concurrent with, UV irradiation. Viability of the cultures was measured daily for 5 days. When Neo6 cells were induced with heavy metals at the same time as UV irradiation, greater than 90% of the cells died within 2 days (Fig. 1). Neo6 cells that were not induced with heavy metal went into a brief lag phase but resumed growth shortly thereafter. If the cultures were induced 24 h prior to UV irradiation (−24 h), there was no cell killing observed (Fig. 1). If induction was maintained from the −24 h time point until the end of the experiment 6 days later, there was little net growth or cytotoxicity. This observation is consistent with our previous finding that Rep expression in Neo6 cells inhibits cell proliferation [41]. When the inducing agent was present from the −24 h time point to the time of UV irradiation, the growth curve looked very similar to the cells that had not received any heavy metal induction.

In the control Neo5 cells, there was some toxicity from the combination of UV and the addition of heavy metal salts at the time of irradiation (Fig. 1). One day after treatment, the Neo5 cells were not affected whereas Neo6 cells displayed greater than 50% cell killing. By the second day 75% of the

Table 1
[^{3}H]Thymidine incorporation in Rep-expressing cell lines

	Neo5	Neo6	Neo40
Untreated	4 508 ± 732	20 805 ± 3 770	12 980 ± 5 155
HM treated	9 129 ± 398	8 403 ± 1 139	3 158 ± 753

Cell lines were treated with heavy metal salts (HM) for 72 h and then pulse-labeled for 4 h. Cultures were harvested and [^{3}H]thymidine incorporation into DNA was determined by TCA precipitation and scintillation counting. The results are derived from triplicate assays.

Neo5 cells remained viable whereas greater than 90% of the Neo6 cells were killed. By 4 days Neo5 viability dropped to 45% but these cells eventually recovered whereas the entire Neo6 culture had been killed. In the other treatment regimens, there was little effect on the viability of the Neo5 cultures. These results indicate that the combined effects of Rep expression and UV irradiation result in cell death.

3.3. Induction of apoptosis-like characteristics by Rep expression and UV irradiation

UV irradiation induces cellular responses that lead to either apoptosis, or DNA repair and cell survival (reviewed in [44]). Nuclear condensation, degradation of nuclear DNA and maintenance of membrane integrity are hallmarks of apoptosis. To determine whether the combination of Rep expression and UV irradiation elicits similar responses, cultures were treated as described in Fig. 1 and stained with acridine orange 16 h after UV irradiation and heavy metal induction. Fig. 2 shows Neo5 and Neo6 cells that were UV irradiated and treated with heavy metal at the time of irradiation. Neo5 cells maintained a normal morphology, whereas the Rep-expressing Neo6 cells changed dramatically. Neo6 cell killing was evident 16 h after treatment. The cells had shrunk and there were acridine orange-stained vesicles in the cytoplasm. The nuclei were invaginated, stained more intensely and there were blebs on the nuclear envelope. These effects were only seen in Neo6 cells that were concomitantly irradiated and heavy metal treated. None of the other treatment regimens resulted in morphological alteration of the cells (results not shown).

To determine whether the integrity of the cell membrane was maintained, Neo5 and Neo6 cells were irradiated and induced with heavy metal as described above. 16 h later, when cytopathic effects were observed, the cells were stained with PI prior to fixation. No staining of the nuclei in any of the cultures was observed (results not shown). If the cell membrane had become porous, as occurs in cellular necrosis, the nuclei should have become accessible to the hydrophobic PI. If the cultures were subjected to fixation prior to PI staining, there was obvious nuclear staining (results not shown). These results suggest that Rep expression and UV irradiation did not alter the cell membrane and that the porosity observed in necrosis was not seen in these cultures.

Coincident with nuclear condensation during apoptosis is activation of endogenous nucleases that attack the inter-nucleosomal linker region in chromatin. In order to determine whether DNA degradation occurred in our UV-irradiated cultures, we treated cells as they were treated in Fig. 1 and chromosomal DNA was isolated 16 h later. The isolated DNA was submitted to agarose gel electrophoresis. Although most of the cells in the heavy metal-induced and UV-irradiated Neo6 cultures had condensed nuclei and were dying, there were no obvious signs of DNA fragmentation to nucleosomal sized fragments (Fig. 3). As a marker for where nucleosomal DNA would migrate in the gel, Neo6 nuclei were isolated and treated with micrococcal nuclease prior to DNA isolation. The lack of a DNA ladder pattern

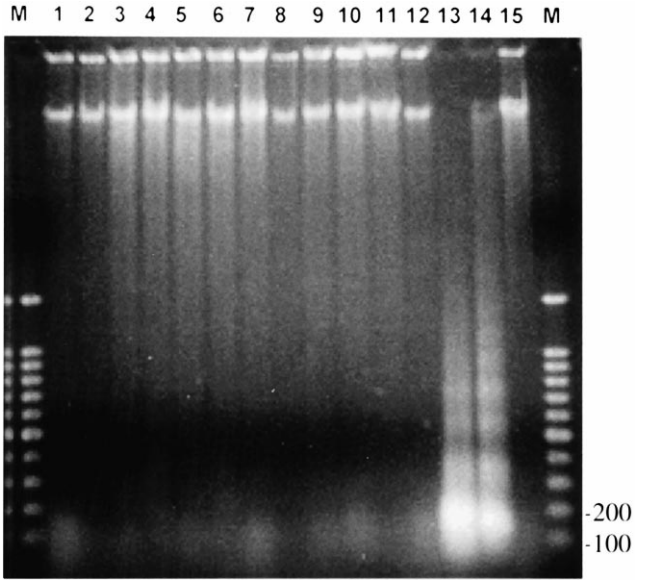

Fig. 3. DNA fragmentation assay. Total cellular DNA was isolated from cells 16 h after treatment with UV light and/or heavy metals (HM), separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Neo5 cells (lanes 1–6) and Neo6 cells (lanes 7–13) were treated as follows: no UV, HM (lanes 1 and 7); no UV, no HM (lanes 2 and 8); UV, HM added 24 h prior to UV and replaced with normal medium after UV treatment (lanes 3 and 9); UV, HM added 24 h prior to UV and maintained until harvest (lanes 4 and 10); UV, HM added immediately after UV (lanes 5 and 11); UV, no HM (lanes 6 and 12). Lanes 13, 14 and 15 contain DNA samples from micrococcal nuclease-treated nuclei from untreated Neo6 cells to serve as nucleosomal DNA size markers. Lane M contains 100 bp ladder DNA marker.

in any of the Neo5 or Neo6 DNA samples indicates that cell killing is occurring without internucleosomal cleavage.

3.4. The effect of Rep expression in UV-irradiated cells on cell cycle and apoptosis-related proteins

In normal cells, UV irradiation induces an increase in the level of the p53 protein [44,45]. A cell cycle checkpoint is realized upon p53 expression enabling the cell to repair its DNA. Alternatively, p53 triggers an apoptotic response. One of the primary effectors of cell cycle arrest is $p21^{Cip}$, an inhibitor of cyclin-dependent kinases [46]. *p21* gene expression is regulated by p53 and $p21^{Cip}$ activity has been detected in normal cells after irradiation, but not in cells that lack p53 [47]. The mechanism whereby p53 induces apoptosis is unresolved. Other cellular targets of p53 regulation include the apoptosis-regulating *bax* and *bcl-2* genes. Bcl-2 is a channel forming protein responsible for maintenance of mitochondrial function [48]. Bcl-2 functions by forming heterodimers with other Bcl family members including the apoptosis-inducing Bax protein. Over-expression of Bcl-2 protects cells from apoptosis [49]. The *bcl-2* promoter is not believed to be transcriptionally affected by p53 but the 5′ untranslated region of the mRNA has been reported to confer negative regulation by p53 [50]. The *bax* promoter contains p53 response elements and is transcriptionally activated by the p53 protein [51]. Thus p53 is believed to enhance apoptosis in response to DNA damage through regulation of the Bcl family of proteins.

To determine whether Rep expression and UV irradiation affect these apoptosis/cytostatic proteins, immunoblot analyses were performed on UV-irradiated and heavy metal-treated Neo5 and Neo6 cultures. The cultures were treated as described in Fig. 1 and were harvested either 16 or 24 h after UV irradiation. As expected, Rep78 and Rep52 expression was observed only in Neo6 cells that had been treated with heavy metal salts (Fig. 4, lanes 7, 9–11). However, Rep protein levels were dramatically reduced in cultures that were UV irradiated and heavy metal induced at the same time (Fig. 4, lane 11). Immunoblots to detect p53 revealed that the only change in p53 levels was in cultures that were UV irradiated and heavy metal induced at the same time

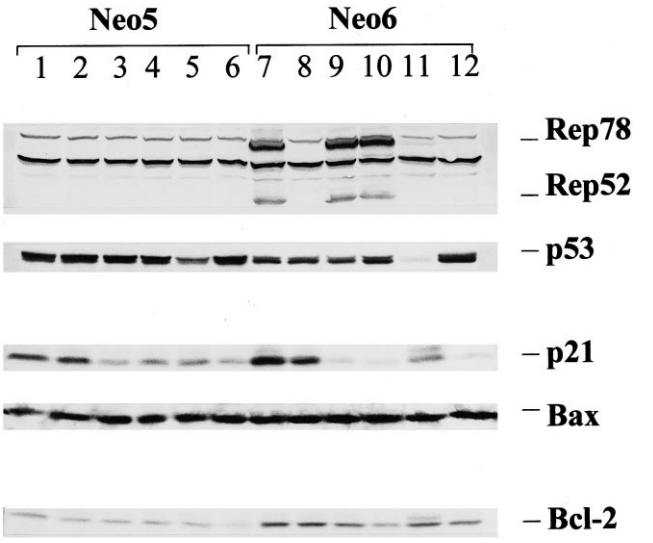

Fig. 4. Protein analyses of Neo5 and Neo6 cells. Immunoblot analyses were performed on whole cell extracts of cultures treated with various combinations of heavy metals (HM) and UV irradiation. Neo5 cells (lanes 1–6) and Neo6 cells (lanes 7–13) were treated as follows: no UV, HM (lanes 1 and 7); no UV, no HM (lanes 2 and 8); UV, HM added 24 h prior to UV and replaced with normal medium after UV treatment (lanes 3 and 9); UV, HM added 24 h prior to UV and maintained until harvest (lanes 4 and 10); UV, HM added immediately after UV (lanes 5 and 11); UV, no HM (lanes 6 and 12). The heavy band running between Rep78 and Rep52 and the lighter band located just above Rep78 are non-specific bands detected by this Rep antibody preparation.

(Fig. 4, lane 11). The drop in p53 and Rep protein levels is surprising but consistent with nuclear breakdown, which is occurring in these cultures. p53 levels were not altered as a result of any of the other treatment regimens.

Analysis of $p21^{Cip}$ levels revealed that there were decreases in both cell types whenever UV irradiation was used (Fig. 4, lanes 3–6, 9–12). In Neo6 cells, there was a modest increase in $p21^{Cip}$ levels and the appearance of more slowly migrating species of $p21^{Cip}$ in cultures that were treated with heavy metals for only 24 h (Fig. 4, lanes 7 and 11). If, however, heavy metal salts were added to the culture medium 24 h prior to irradiation, the $p21^{Cip}$ level dropped compared to the untreated controls and the more slowly migrating forms were not observed (Fig. 4, lanes 9 and 10). These other forms of $p21^{Cip}$ were not detected in UV-irradiated Neo6 cells in the absence of heavy metal treatment (Fig. 4, lane 12).

Analysis of the extracts for changes in Bax expres-

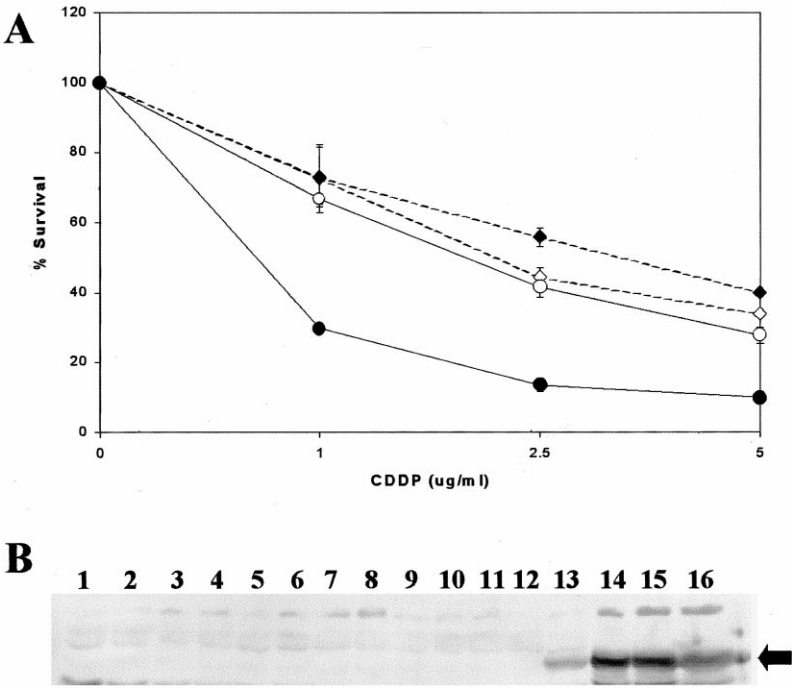

Fig. 5. *cis*-Platin and Rep expression are cytotoxic. (A) Neo5 and Neo6 cells were incubated with increasing concentrations of *cis*-platin (CDDP) and viability assays were performed 3 days after treatment. Neo5 cells were treated with heavy metal salts (dashed line, filled diamonds) or left untreated (dashed line, open diamonds). Neo6 cells were treated with heavy metal salts (solid line, filled circles) or left untreated (solid lines, open circles). Each point is derived from an average of triplicate cultures and the experiment was repeated twice. (B) Neo5 and Neo6 cells were treated with CDDP and heavy metal salts and harvested 3 days later. Cell extracts were prepared and analyzed by immunoblotting with Rep-specific antibodies. Neo5 (lanes 1–8) and Neo6 (lanes 9–16) cells were treated with increasing concentrations of CDDP as follows: 0 μg/ml (lanes 1, 5, 9, 13), 1 μg/ml (lanes 2, 6, 10, 14), 2.5 μg/ml (lanes 3, 7, 11, 15), 5 μg/ml (lanes 4, 8, 12, 16). Cultures were left untreated (lanes 1–4, 9–12) or treated with heavy metal salts (lanes 5–8, 13–16). The location of Rep78 is indicated by the arrow at the right.

sion revealed that there was no change in the level of this apoptosis-inducing protein (Fig. 4) in either cell line after any of the treatments. Bcl-2 levels were also not affected in Neo5 cells. In Neo6 cells there was a decrease in the level of Bcl-2 when heavy metals were left on the cells from the −24 h time point until the cultures were harvested (Fig. 4, lane 10). In Neo6 cells that were concomitantly induced with heavy metal and UV irradiated, there was the appearance of a more slowly migrating species (Fig. 4, lane 11). The concurrent heavy metal treatment and UV irradiation of Neo6 cells is the only treatment regimen in which we have seen the slower-migrating Bcl-2 species. This species may be due to post-translational modification and its mobility is very similar to that observed upon Bcl-2 phosphorylation [51]. Phosphorylation of Bcl-2 has been described in several systems and is proposed to play pro-apoptotic [53] and anti-apoptotic [54] roles in Bcl-2 function.

3.5. *cis*-Platinum and Rep expression are cytotoxic

To determine whether another DNA-damaging agent enhances Rep toxicity, we performed a dose-response experiment with *cis*-platinum (CDDP). Neo5 and Neo6 cultures were treated with increasing concentrations of CDDP for 24 h and then the CDDP was removed and the cultures were induced with heavy metal salts (Fig. 5A). Three days later, viability assays were performed. Heavy metal induction of Neo5 cells had no effect on viability at any of the CDDP concentrations. However, heavy metal treatment substantially diminished the viability of Neo6 cells at all three concentrations of CDDP. Heavy metal treatment in the absence of a DNA-damaging agent results in cytostasis rather than cytotoxicity [41]. In these experiments, CDDP was included in the culture medium for 24 h and was then replaced with normal medium lacking, or supple-

mented with, heavy metal salts until viability assays were performed. We observed no differences in cell killing or Rep protein effects if the CDDP was kept in the medium for the duration of the experiment (results not shown). These results indicate that Rep protein expression enhances CDDP-mediated cell killing. To verify that Rep protein is expressed in the presence of CDDP, Western immunoblot analyses were performed. As expected, Rep78 is induced to a high level in response to heavy metal induction and CDDP did not induce expression in the absence of Zn and Cd salts (Fig. 5B). It is worth noting that the combination of heavy metal salts and CDDP results in an increased level of Rep78 protein (Fig. 5B, lanes 13 and 14). It is possible that repair of the CDDP-induced lesions results in chromatin remodeling in the vicinity of the *rep* gene which could lead to an increase in transcription rate. However, we have not yet determined the reason for this increase.

4. Discussion

The cytotoxic effects of the parvoviruses have been known for many years. Numerous studies have linked viral non-structural gene expression with cytostasis or cytotoxicity. The mechanism by which parvoviral non-structural proteins affect host cells is only recently coming under extensive study. In this report we have used an AAV Rep protein-expressing cell line to study the anti-proliferative effects of the Rep protein and DNA-damaging agents. Our results demonstrate that expression of the AAV Rep proteins and concurrent UV irradiation result in apoptosis-like cell killing. We also found that DNA damage from *cis*-platinum caused cell killing when accompanied by Rep expression. This Rep/DNA damage-mediated cell killing bears some of the characteristics of apoptosis including maintenance of cell membrane integrity and nuclear condensation fragmentation. However, we did not observe any obvious DNA degradation and generation of a nucleosomal repeat pattern in chromosomal DNA. There are a number of reports in the literature that Zn^{2+} inhibits the endogenous nucleases that are activated in apoptosis [55]. One possibility why a nucleosomal repeat pattern was not observed is that 100 μM $ZnCl_2$ was used to induce Rep expression. Thus in inducing Rep expression we may be inhibiting apoptotic nucleases. Another possibility is that accumulation of Rep proteins in the nucleus of the cell inhibits endogenous nuclease action. We are currently trying to determine which of these two possibilities explains why DNA degradation is not detected during what is apparently an apoptotic event.

When Rep expression preceded UV irradiation by 24 h, we did not see any enhancement of cell killing. This is a surprising result given the very rapid cytotoxicity observed when Rep expression is induced immediately after irradiation. One reason for this result may be explained by the DNA synthesis data shown in Table 1 and our previous results describing an S-phase block in Neo6 cells when Rep is turned on [41]. Rep expression prevents the cell from cycling through S-phase. Therefore the cell has time to repair damaged DNA before resuming the cell cycle. In this way Rep may function similarly to p53 in that upregulation of p53 in response to DNA damage mediates a G_1 block to allow for DNA repair to occur.

UV irradiation results in increased p53 function due to an increased level of the protein [45,56]. This increase allows for DNA repair and cell survival or it can trigger apoptosis. The UV fluency used in this work was not lethal and also did not induce an increase in p53 levels. Nor did heavy metal salts or Rep expression affect p53 levels. The only change in p53 levels was observed in cultures that were dying. In these cultures p53 was significantly decreased. A similar decrease was also observed in the levels of Rep protein. We believe that the loss of these proteins can be explained by nuclear degradation. Although we are not aware of any reports in the literature describing p53 as a target for protease action in apoptotic cells, potential caspase cleavage sites are found in the primary sequence of p53 as well as Rep78. Thus it is quite possible that both of these proteins are protease targets. That there was no increase in p53 levels in responses to Rep or UV light suggests that the cell killing observed in Rep-expressing, UV-irradiated cells is due to p53-independent mechanisms.

Analysis of other apoptotic/cytostatic proteins revealed some interesting observations. There was a modest increase in $p21^{Cip}$ expression in Neo6 cells after 24 h of heavy metal treatment. Given the role of $p21^{Cip}$ in mediating a G_1 cell cycle block, its in-

crease in response to Rep expression would certainly fit with the results in Table 1. These observations are interesting in light of a recent report that AAV infection of primary human cells resulted in an increase in $p21^{Cip}$ expression at the protein and mRNA levels [35]. In primary human cells, AAV stimulated $p21^{Cip}$ mRNA within the first 6 h, suggesting that the increase was due to the infecting virion rather than to de novo expression of Rep proteins and subsequent trans-activation of the *p21* gene. However, until AAV *rep* gene mutants are tested for their ability to induce $p21^{Cip}$ expression in primary human cells, it is difficult to verify that Rep protein expression is not involved. In our studies, UV irradiation concurrent with Rep expression (and to a lesser extent Rep expression after only 24 h) resulted in the appearance of more slowly migrating species of $p21^{Cip}$. If, however, the Rep-expressing cultures were not harvested for an additional 24 h, the increased level of $p21^{Cip}$ and the slower migrating species were no longer detectable. The new $p21^{Cip}$ species suggests that they may be post-translationally modified versions of the protein. Ubiquitination of $p21^{Cip}$ has been described but the slight decrease in mobility is unlikely due to ubiquitin, which would add 8 kDa to the size of the protein. The modest decrease in mobility in gel electrophoresis suggests that the post-translational modification may be due to a small moiety such as phosphorylation.

In UV-irradiated, Rep-induced cultures we observed the appearance of a new species of Bcl-2. In cultures that were UV irradiated and immediately induced to express the Rep proteins, a more slowly migrating Bcl-2 was observed. The migration of the new species is very similar to that of phosphorylated Bcl-2 [52]. Although we have not yet been able to determine whether Bcl-2 phosphorylation is increased in heavy metal-induced and UV-irradiated Neo6 cells, phosphorylation of Bcl-2 has been linked to the apoptotic process. Phosphorylation of Bcl-2 has been described in several systems and both pro-apoptotic and anti-apoptotic roles have been attributed to this modification [53,54].

In conclusion, we have provided evidence that the AAV Rep proteins induce apoptosis-like killing in cells that have been challenged by DNA-damaging agents. It is worth noting that these exogenous treatments create a favorable environment for helper virus-independent AAV replication. Induction of apoptosis in AAV-infected, permissive cells would provide a means to release progeny in the absence of a co-infecting helper virus. Apoptosis in B19-infected fetal erythroid precursors has been demonstrated [57] and cell killing via an apoptosis pathway has been attributed to the NS1 protein [58]. One possible mechanism of parvovirus-mediated cell killing could involve non-structural protein binding and subsequent nicking of cellular DNA. Evidence has been provided demonstrating that nicking of cellular DNA is involved in blocking DNA replication [59]. Several cellular DNA binding sites for AAV Rep proteins have been identified [60] and it will be interesting to determine whether these sites are targets of Rep-specific nicking.

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