In Vitro Adenoviral Vector p53-mediated Transduction and Killing Correlates with Expression of Coxsackie-Adenovirus Receptor and \( \alpha_5\beta_5 \) Integrin in SUDHL-1 Cells Derived from Anaplastic Large-Cell Lymphoma

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ABSTRACT

Adenoviral vector-mediated p53 expression induced apoptosis is a well established gene therapy approach that has been evaluated extensively in epithelial tumors but only recently in lymphoid malignancies mainly due to the known resistance of the lymphoid lineage to adenovirus infection. Recently, it was shown that this resistance is not absolute and that cell lines derived from anaplastic large cell lymphoma (ALCL) and some other lymphoid malignancies are efficiently transduced by adenoviral vectors. Normal circulating T lymphocytes do not express coxsackie-adenovirus receptor (CAR) and \( \alpha_5\beta_5 \) integrins and are relatively resistant to infection by adenovirus. These molecules serve as receptors for adenovirus entry into the cells. ALCL-derived SUDHL-1 cells were evaluated for transduction efficiency and expression of p53 after infection with an adenoviral vector containing wild-type p53 (AdWTp53). Cells derived from ALCL and circulating mononucleated cells (MNCs) were also evaluated for expression of CAR and \( \alpha_5\beta_5 \) integrins. AdWTp53-mediated expression of p53 resulted in p21/WAF1 induction, G1 arrest, and apoptosis in SUDHL-1 cells. The expression of CAR and \( \alpha_5\beta_5 \) integrin was high in SUDHL-1 cells and comparable to levels observed with epithelial tumor cells, but it was absent in MNCs. The susceptibility to adenoviral vector transduction of the tumor-derived cells implies an important biological difference between them and circulating MNCs, possibly underlying the malignant transformation that ALCL cells undergo. Further studies will be required to evaluate this initial observation in more cell lines and tissue derived from ALCL.

INTRODUCTION

Apoptosis is a genetically controlled programmed cell process that culminates with chromatin condensation, DNA fragmentation, and cell death (1). Slowed or absent apoptosis of cells can lead to tumor formation. The protein product of the tumor suppressor gene p53 is a bivalent nuclear phosphoprotein that can arrest cell growth via the cyclin-dependent kinase inhibitor p21/WAF1, thereby allowing the cells to repair their DNA damage. Alternatively, p53 can also induce apoptosis by transcription dependent and independent pathways (2).

Shaw et al. (3) initially showed the in vivo induction of apoptosis of tumors derived from human colon carcinoma cells by plasmid-mediated wild-type p53 expression. Recently, adenoviral vector-mediated p53 gene therapy has been extensively studied in epithelial solid tumor models (4–10). The exogenous expression of p53 by adenoviral vector has, however, not been well explored in lymphoid malignancies (11). Lymphoid models have not been considered promising perhaps because of the poor transduction of normal circulating lymphocytes by adenoviral vectors (12). Normal T lymphocytes isolated from the peripheral blood do not express the known adenoviral receptors. The CAR and \( \alpha_5\beta_5 \) integrins are two cell membrane components that interact with the adenoviral capsid fiber protein and the penton base, respectively. The fiber protein initially attaches to the cellular receptor, and the penton base binds to \( \alpha_5\beta_5 \) integrins facilitating the viral particle internalization into the cells via receptor-mediated endocytosis (13, 14). However, we previously demonstrated very efficient \( \beta \)-galactosidase gene expression in several cell lines derived from lymphoid malignancies after infection with adenoviral vectors (15). In a more recent study, we have shown efficient killing of cells derived from a broad spectrum of lymphoid malignancies induced by two adenoviral vectors expressing a thymidine kinase from herpes simplex-1 and the wild-type p53 tumor suppressor gene (16).

In the present study, the transfer and expression of the wild-type p53 tumor suppressor gene by an adenoviral vector were evaluated in SUDHL-1 cells derived from ALCL. Analysis

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3 CAR, coxsackie-adenovirus receptor; ALCL, anaplastic large cell lymphoma; MNC, mononucleated cell; MOI, multiplicity of infection; TUNEL, terminal deoxynucleotidyl transferase; PI, propidium iodide; MoAb, monoclonal antibody.
was conducted to define the mechanism of induced cell death in SUDHL-1 cells. Expression of p53 after adenoviral vector gene transfer induced SUDHL-1 cells to undergo apoptosis. Apoptosis occurred after the induction of p21/WAF1 expression and G1 phase cell cycle arrest.

We also evaluated these cells for the presence of receptors that explained high efficiency transduction of a lymphoma cell by an adenoviral vector. Thus, SUDHL-1 and circulating blood MNCs were screened for the expression of CAR, αβ5, and αβ3 integrins. The efficiency of adenoviral vector gene transfer into SUDHL-1 cells was similar to the efficiency of epithelial carcinoma cells as previously shown (e.g., HELA; Ref. 15).

The efficiency of adenoviral transduction in SUDHL-1 correlates strongly with an unexpected, high level of expression of the CAR receptor and αβ3 integrin, which is similar to epithelial cell lines.

**MATERIALS AND METHODS**

**Cell Lines.** Human cell line SUDHL-1 was kindly provided by Dr. S. Morris, St. Jude Children’s Research Hospital and Dr. S. Smith, University of Kansas (17). HELA S3 (cervical carcinoma) and A549 (lung carcinoma) cells were obtained from American Tissue Culture Collection, Rockville, MD. Circulating MNCs were obtained from a single donor by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) after informed consent was given, and they were kept in culture. The cell phenotype of the MNCs was not characterized. All cells were cultured in DMEM and RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10–15% heat-inactivated fetal bovine serum, 2 mmol/l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Recombinant Adenovirus Infection.** SUDHL-1 cells (2.5–5 × 105) were exposed to AdNull (adenoviral vector backbone without cDNA insert) and AdWTP53 expressing p53 (AdWTP53; Ref. 5; 100 MOI) in 250 μl of PBS for 1 h at 37°C. Cells that were used were at least 95% viable by trypsin blue (Sigma, St. Louis, MO) exclusion assay. Cells were then seeded in a 60-mm Petri dish with media at 2% fetal bovine serum and further incubated at 37°C for 14–48 h.

**Flow Cytometry Analysis of p53, p21/WAF1, and Apoptosis in AdWTP53-Infected Lymphoma Cells.** To evaluate for p53 and p21/WAF1 expression, cells (5 × 105) were washed 14–18 h after infection with PBS and fixed with 70% methanol at 20°C for 20 min as previously described (18). Cells were washed and then incubated with either anti-p53 monoclonal antibody (1 μg/ml, Ab-6, Oncogene Research Products, Cambridge, MA) or anti-p21/WAF1 monoclonal antibody (1 μg/ml, Ab-1, Oncogene Research Products). The cells were next incubated with secondary fluorescein conjugated (FITC) goat antimouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Isotypes IgG2 and IgG1 (ICN Biomedical, Inc, Aurora, OH) were used as matched controls for anti-p53 and anti-p21/WAF1, respectively. Samples were analyzed with an Epics XL cytometer (Coulter, Miami, FL), and single parameter histograms were obtained. The percentage of the difference of the mean intensity of FITC fluorescence between the monoclonal antibody and the matched isotype was obtained for each sample.

Cells (5 × 105) in triplicate were washed 24 and 48 h postinfection with PBS, fixed with methanol, and evaluated by TUNEL analysis. The TUNEL assay was performed on 24 and 48 h postinfection cells using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions.

Cells were analyzed for fluorescence by using the flow cytometer as above. For each group (mock, AdNull, and AdWTP53), one way ANOVA of the log of the mean intensity (=Σ log to lin [channel number] × count in the channel/area) of FITC fluorescence was obtained. Data analysis by the Tukey test (SigmaSTAT) was used to find the significance of differences in mean fluorescence between the three test groups for TUNEL analysis.

**Cell Viability (Cell Death) Analysis.** For the cell viability analysis, aliquots of SUDHL-1 cells were analyzed in triplicate by flow cytometry 24 and 48 h after infection using the PI staining (Coulter Reagents Kit, Coulter) according to the manufacturer’s instructions. All samples were analyzed on the Epics XL cytometer. This analysis was based on the use of PI staining of dead cell compared to cells still viable. For each group (mock, AdNull, and AdWTP53), one way analysis of the log of the mean PI intensity and analysis of significance of the difference among the groups were obtained by using the Tukey test, as specified above for TUNEL analysis.

**DNA-Content (Cell Cycle) Analysis.** For DNA-content analysis, SUDHL-1 cells (5 × 105) were washed in 1 × PBS and stained with PI in triplicate 10, 14, 18, and 24 h after infection and analyzed for percentage of cells in each phase of the cell cycle using an Epics XL cytometer. Histograms of the percentage of cells in the G1, S, and G2 phase were expressed as mean ± SD. Each sample was performed in triplicate.

**Flow Cytometry Analysis of CAR and Integrin Expression.** For CAR expression analysis, HELA S3, A549, SUDHL-1 cells, and circulating MNCs (2.5–5 × 105) were washed once with PBS and then incubated for 30 min at room temperature with RcmB monoclonal antibody (2 μg/ml), a generous gift from Dr. L. Philipson, Karolinska Institute, Stockholm, Sweden (13). Cells were washed with PBS and incubated for 30 min at room temperature with a secondary FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Isotype IgG1 (ICN, Biomedical) was used as a matched control for each sample.

For integrin expression analysis, samples of the same cells mentioned above (2.5–5 × 105) were initially incubated with 25 μg/ml of anti-αβ3 (MAB 1961, Chemicon International, Temecula, CA) and anti-αβ5 (MAB1976, Chemicon International) monoclonal antibodies for 30 min at 4°C. Cells were washed with PBS and incubated with a secondary FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Matched isotype control for each sample was used and analyzed as above.

All samples were analyzed using an Epics XL cytomter, and a single parameter histogram was obtained. The percentage of the difference of the mean of the FITC fluorescence intensity between the sample and the matched control was obtained.
Fig. 1  Flow cytometry analysis of p53, p21/WAF1, apoptosis, and cell viability in AdWTp53 transduced ALCL cells. SUDHL-1 cells after 14 and 18 h of infection were fixed with methanol and stained with anti-p53 or anti-p21/WAF1 MoAb and FITC-conjugated antibody and analyzed by flow cytometry. IgG2 and IgG1 isotypes were used as matched controls, respectively. A and B, C and D, the expression of the two proteins as the most representative histogram (gray) matched with their respective controls (white) from ALCL cells mock-infected and infected by AdWTp53 (adenoviral vector expressing p53). The difference of the mean FITC fluorescence between the sample and the control are given for each pair. SUDHL-1 cells 48 h after infection were fixed with methanol and prepared for TUNEL analysis. Aliquots of the cells after 48 h of infection were stained with PI without fixation in methanol for cell viability. All samples were analyzed by flow cytometry. E and F, the most representative flow histogram from a triplicate of mock-infected cells, cells infected with either 100 MOI of AdNull (adenoviral backbone) or AdWTp53.
RESULTS

Expression of p53, p21/WAF1, Apoptosis, and Cell Death Determined by Flow Cytometry. p53 protein expression in the SUDHL-1 cells after adenoviral vector infection was defined by flow cytometry analysis. Mock-infected (Fig. 1A) and AdNull-infected (not shown) cells showed 27% and 20% of p53 expression, respectively. The level of p53 expression increased to 86% in cells infected by AdWtp53 14 h after infection (Fig. 1B). The expression of p21/WAF1 was absent in mock-infected (Fig. 1C) and AdNull-infected (not shown) cells, but it raised to 65% only in cells infected by AdWtp53 18 h after infection (Fig. 1D). Although SUDHL-1 cells express p53, p21/WAF1 was efficiently expressed only in cells transduced by AdWtp53.

To measure the extent of apoptosis occurring in the cells as a consequence of the overexpression of p53 and sequential expression of p21/WAF1 induced by AdWtp53, we analyzed cells 24 and 48 h after infection by flow cytometry after TUNEL assay and PI staining. Aliquots of the cells from the infections and mock infection were split and then processed for TUNEL assay or PI staining. PI staining was performed before fixation and permeabilization with methanol to reduce the false positive staining of cells.

A strong correlation was found between infection by AdWtp53 and high level FITC fluorescence with the TUNEL assay compared to the mock- and AdNull-infected cells (Fig. 1E). The PI fluorescence analysis showed an identical pattern with strongly positive labeling in the same AdWtp53-infected cell population (Fig. 1F). The results were highly significant for both TUNEL (P = 0.01) and PI (P < 0.001) in AdWtp53 cells versus mock and AdNull. No significant difference was noted between mock- and AdNull-infected cells for either assay (Fig. 2, A and B). The estimated percentage of apoptotic cells (TUNEL assay) and dead cells (PI staining for cell viability) was 80 and 85%, respectively (data not shown). Thus, cell death is strongly correlated with apoptosis after p53 induced overexpression is followed by p21/WAF1 expression in AdWtp53-infected SUDHL-1 lymphoma cells. This effect peaked at 48 h and was only minimal within the 24 h (data not shown).

DNA-Content (Cell Cycle) Analysis. The effects of AdWtp53 adenoviral vector infection on the cell cycle of the SUDHL-1 cell line was determined by analyzing DNA content of infected cells by flow cytometry at varying times after infection. Cells were analyzed from 10 to 24 h after infection. Over this time interval infection, the fraction of cells in the G1 phase showed a steady increase only in transduced AdWtp53 SUDHL-1 cells. No change in the G2 phase was observed (Fig. 3A). In concert, the fraction of cells in the S phase steadily decreased only in the AdWtp53-infected cells (Fig. 3B). None of the three test populations showed a significant change over this time period in the fraction of cells in the G2 phase (Fig. 3C).

Flow Cytometry Analysis of CAR and Integrin Expression. The monoclonal antibody RcmB binds to the well-defined human coxsackie virus B and adenovirus receptor (19). SUDHL-1 exhibited strongly positive CAR expression, similar to HELA S3 by flow cytometry analysis. Circulating MNCs were confirmed to be negative for CAR expression (Fig. 4A).

Flow cytometry analysis using monoclonal antibodies against α5β1 and α6β1 showed that integrins demonstrated a higher level of expression of α5β1 on the cell membrane of SUDHL-1 compared to α6β1. This expression level was more similar to the pattern of expression of the epithelial cell lines HELA S3 than A549, which express significant levels of both types of α5β integrins (20). Circulating MNCs showed no expression of integrins (Fig. 4, B and C).

DISCUSSION

Several studies have previously evaluated the use of adenoviral vectors to transduce lymphoid cells using marker genes such as β-galactosidase (16, 21, 22). These data demonstrated that the majority of lymphoid cells or tissues were poorly transformed by adenoviral vectors. More recent investigations have focused on effector genes like p53 (16), herpes simplex thymidine kinase (16), and interleukin-2 (23, 24). Although these studies did show some efficiency, they required a high MOI of the adenovirus (21), prolonged time of infection (23, 24), or stimulation of cell membrane integrin expression with cytokines (21). A recent study has for the first time shown...
efficiency of cell killing in cells derived from multiple myeloma mediated by adenovirus-expressed thymidine kinase and ganciclovir. In this study, CAR and αβ5 integrin expression were shown to correlate with gene transduction in those cells (25). Since our first observation that JB6 cells derived from ALCL were transduced by low MOI of adenovirus expressing β-galactosidase gene, other cell lines derived from lymphoid malignancies were tested (15). The initial observations with ALCL were extended to SUDHL-1 and UCONN-L2 cells derived from the same type of lymphoma. These data suggested a common molecular mechanism in cells derived from this non-Hodgkin’s lymphoma that permits efficient adenoviral vector transduction. Follow-up experiments with JB6 cells showed effective in vitro killing after transduction by an adenoviral vector containing a herpes simplex thymidine kinase gene and treatment with ganciclovir (26). A more recent study, which used adenoviral vectors in a broader spectrum of lymphoid malignancies, demonstrated the efficacy of killing with p53 or thymidine kinase (16). Two important concepts were established in these studies: (a) the resistance to adenoviral transduction is not absolute in the lymphoid lineage; and (b) ALCL requires a lower MOI compared to other lymphoid cell studied to achieve efficient gene expression. The cell lines derived from ALCL cells were more efficiently transduced like epithelial carcinoma cells. Further study of this cell line as a model was warranted to understand these observations.

Adenoviral transfer expression of p53 for solid tumor gene therapy has been evaluated for a variety of tumors derived mainly from head and neck, lung, kidney, prostate, breast, cervix, and ovary (4–11). Our data shows endogenous p53 expression in SUDHL-1 cells. This finding is consistent with a recent study that found >60% of ALCL tumors from fresh tissue samples expressing significant levels of p53 by immuno-histochemical analysis and only one ALCL lesion carrying a p53 gene mutation (exon 5; Ref. 27). The endogenous p53 status has been shown to be a determinant of AdWTp53 cytotoxicity (5). Our in vitro data show that exogenously induced AdWTp53-mediated expression of wild-type p53 is feasible in ALCL cells and induces cell death through apoptosis. Changes in the cell cycle after AdWTp53 transduction of SUDHL-1 cells correlate well with prior data that show that p21/WAF1 expression is necessary to induce G1 arrest and regulate the G1-S

![Fig. 3](attachment:image.png)

Fig. 3 Cell cycle analysis. SUDHL-1 cells were stained with PI at different times after mock infection with AdNull or AdWTp53 and analyzed by flow cytometry. Note the increase with time in the G1 population with a decline in the S phase population after infection with AdWTp53. A, B, and C, histograms of the percentage of cells from triplicate experiments expressed as mean ± SD.
Fig. 4  Flow cytometry analysis of the CAR, α,β₅, and α,β₃ expression. A, cells were stained with anti-RcmB (CAR) MoAb and secondary FITC-conjugated antibody as described in “Materials and Methods” and analyzed by flow cytometry. IgG1 isotype was used as a matched control (white histogram). The histogram (gray) from HELA S3, A549, SUDHL-1, and circulating MNCs are shown. B, cells were stained with anti-α,β₅ MoAb and secondary FITC-conjugated antibody as described in “Materials and Methods” and analyzed by flow cytometry. IgG1 isotype was used as a matched control (white histogram). The histogram (gray) from HELA S3, A549, SUDHL-1, and MNCs are shown. C, cells were stained with anti-α,β₃ MoAb and secondary FITC-conjugated antibody as described in “Materials and Methods” and analyzed by flow cytometry. The IgG1 isotype was used as a matched control (white histogram). The histograms (gray) from HELA S3, A549, SUDHL-1, and MNCs are shown. The difference in percentage of the mean FITC fluorescence between the sample and matched control are given for each cell line.
transition (28) without involvement of the G1 checkpoint (29). TUNEL has been proven to be an accepted and valuable method for the detection of apoptosis (30). TUNEL assay by flow cytometry analysis and cell viability by PI staining of AdWTP53 transduced lymphoma cells clearly established that apoptosis and death occur in 80 and 85% of the AdWTP53 transduced cells.

The susceptibility to adenoviral transduction demonstrated in our results implies an important biological difference associated between ALCL cells and circulating MNC cells. We hypothesized that the presence of higher levels of adenoviral viral receptors might be responsible. ALCL may represent a model to study lymphoid-epithelial transformation, which is likely responsible for the biological behavior of this lymphoma and its high propensity to metastasize to extra-lymphoid tissues (31). The expression of integrins and CAR correlates with the coexpression of the epithelial membrane antigen (32), cytokeratin, and vimentin (33) by ALCL. The natural expression of integrins by SUDHL-1, particularly of the β1 integrin (data not shown), may explain the propensity of this lymphoma type to metastasize. A recent study suggests an important role for this integrin in the metastasis of a murine T-lymphoma cell line (34). The resting normal circulating T lymphocyte does not express significant levels of either CAR (13) or integrins (12). The relative lack of expression correlates with a resistance of inactivated lymphocytes to adenovirus transduction. Our flow cytometry data confirm the lack of expression of CAR and integrins in the circulating MNCs, which include subpopulations of circulating lymphocytes (although the phenotype of these cells was not evaluated in the present study).

We hypothesize that the malignant transformation process of ALCL closely correlates with the expression of the two cell membrane components, CAR and α5β1 integrin, which also function as adenovirus receptors. Thus, the observation of an ALCL cell line being efficiently transduced by adenovirus is an acquired phenotype that may signify mutations associated with the malignant transformation of the cell type. ALCL is also a heterogenous subtype of intermediate grade lymphoma with different cell phenotypes and genotypes (35–37). The tumor heterogeneity may correlate with variation in the expression of CAR and integrins among ALCL tumors. Whether this property is peculiar to ALCL or also occurs in more common lymphoid tumors is as yet unknown. More investigation is warranted to elucidate the underlying molecular mechanism responsible.

Our study shows for the first time the efficiency of an in vitro model of adenoviral-induced apoptosis by overexpression of wild-type p53 in a lymphoma-derived cell line. Another recent study has also documented the expression of apoptosis-related genes in head and neck squamous cell carcinoma undergoing p53 adenoviral-mediated cell death (38).

The question of whether or not our results will support the continued development of gene therapy with adenovirus in ALCL or other lymphomas relies mainly on the documentation of the expression of CAR and integrins in tissue or cells derived from patients with ALCL. Although other investigators have recently shown a strong CAR signal by immunocytochemistry on cryosections derived from melanoma tumor biopsies (39), we have failed thus far to show a specific signal in ALCL-derived tumors (data not shown in collaboration with Dr. D. D. Weisenburger and Dr. P. Aoun, Department of Pathology, University of Nebraska, Omaha, NE). Flow cytometry detection of CAR and integrins has been used by other investigators to show expression in cells derived from the bone marrow of patients with multiple myeloma (25). The lower incidence of ALCL compared to multiple myeloma makes the availability of fresh tissue or cells for flow cytometry more difficult. This may require joint efforts of several centers to obtain sufficient fresh tumors for analysis. We are presently evaluating alternative methods to detect CAR expression in tissue. In an effort to answer the question of feasibility of gene therapy of lymphoma, we have also established an athymic nude mouse model of ALCL (40) to study the potential for in vivo tumor killing with AdWTP53 vectors.

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