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Recombinant E1-deleted adenovirus vector induces apoptosis in two lung cancer cell lines

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Recombinant E1-deleted adenovirus vector induces apoptosis in two lung cancer cell lines. S. Teramoto, T. Matsuse, H. Matsui, E. Ohga, T. Ishii, Y. Ouchi. ©ERS Journals Ltd 1999.
ABSTRACT: Although replication-defective adenoviruses (Ads) are used as vectors for delivering therapeutic genes to cancer cells, various effects of the viruses on the proliferation of lung cancer cells have been reported.

Experiments were carried out to determine whether or not E1-deleted Ad vectors (Ad5-CMV-*lacZ*) affected cell kinetics in two different types of lung cancer cell line *in vitro*.

A dose-dependent relationship was measured between the vector multiplicity of infection (MOI) and the efficiency of *lacZ* gene transfer to lung cancer cells. The growth curves of vector-infected cells were shifted to the right compared with those of vehicle-exposed cells in a vector MOI-dependent fashion. The slowed cell proliferation resulted from both increased cell death and slower cell cycle progression of the vector-infected cells. The morphology of vector-exposed cells revealed apoptotic features including nuclear condensation and fragmented nuclei.

These results indicate that using a higher vector MOI causes a higher gene transfer rate, but may induce apoptosis of infected cells. Although vector-induced apoptosis may be advantageous in inhibiting tumour growth, apoptosis of vector-infected cells may also reduce transgene expression in cancer cells. Minimization of the induction of apoptosis of vector-infected cells is important for the prolongation of the transduction efficiency of Ad vectors.
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Replication-incompetent adenovirus (Ad)-mediated gene transfer to cancer cells is a potentially powerful tool for the *in vivo* delivery of therapeutic genes, such as *p53*, to lung cancer cells [1, 2]. Preclinical gene therapy trials are underway to test the efficacy and safety of Ad vectors in patients with lung carcinoma [3]. Since Ad vectors efficiently transduce foreign genes into a wide variety of cell types and into proliferating as well as nonproliferating cells, they are attractive candidates for the gene therapy of lung carcinoma cells, which are characterized by heterogeneity of the cell cycle [4, 5].

However, several limitations of controlling lung cancer growth with *in vivo* gene therapy using Ad vectors should be considered. Ad vectors are known to cause extensive host immune and inflammatory responses [6–8], and the immune response may be responsible for the loss of transgene expression [9]. Furthermore, Ad vector infection may affect target cell proliferation and cell death. Although ZHANG *et al.* [10] have demonstrated that the growth of human non-small cell lung carcinoma cells (H1299) is not affected by infection with Ad5-RSV-luciferase at a multiplicity of infection (MOI) of 100 plaque-forming units (PFU)·cell^{-1}, KATAYOSE *et al.* [11] have reported that an Ad vector and a replication-deficient Ad dl312 inhibit the proliferation of vascular smooth muscle cells at a MOI of ≥800 PFU·cell^{-1}. It has been found that Ad vectors (Ad-CMV-*lac*Z and Ad-CB-CFTR) perturb proliferation of human airway epithelial cells causing apoptosis and slowing the cell cycle [12]. These observations suggest that higher MOIs of Ad vectors may be toxic to normal and/or cancer cells. The cell kinetics of Ad vector-infected lung cancer cells have, however, not been completely elucidated. Although wild-type Ad infection elicits a potent cytopathic effect in the infected cells [13, 14], E1 gene-deleted Ad vectors are theoretically nonreplicating, and should not induce lytic infection of host cells. It is not known, however, whether other Ad genes, present in Ad vectors, affect the proliferation of tumour cells after Ad vector-exposure.

The present study was designed to determine whether or not E1-deleted Ad vectors (Ad5-CMV-*lacZ*) affected cell growth in two different types of lung cancer cell line: a lung adenocarcinoma cell line and an epidermoid cell line.

Methods

Adenoviral vector

A replication-defective adenoviral vector based on the human Ad5 serotype (hAd5) was used for the study. E1 and E3 sequences were deleted from the hAd5-CMV-*lacZ* vector and replaced with a gene containing the cytomegalovirus (CMV) promoter and a cytoplasmic *lacZ* gene at the site of the E1 deletion of Ad [15–18]. The Ad vector

was propagated in HEK293 cells, purified by means of CsCl density gradient ultracentrifugation, and stored at -70°C until used for infection of lung cancer cells. The titre of the Ad vector (in transducing units (TU)·mL^{-1}) was determined from the number of *lacZ* gene-expressing HEK293 cells·mL vector-1 by means of histochemical X-Gal staining [15–18]. The vector was used at titres of 1×10^{11}–10×10^{11} (TU·mL^{-1}) in the experiments. The ratio of TUs to viral particle number (as measured by optical density at 260 nm) was approximately 1:20–50.

Cell culture

In this study, a lung adenocarcinoma cell line (H1437) and an epidermoid cell line (A431) were used. H1437, originating from human lung cancer pleural metastases, was a generous gift from H.K. Oie (National Cancer Institute, Bethesda, MD, USA) [19]. The cells were fed on alternate days with RPMI 1640 containing 10% foetal bovine serum (FBS), penicillin and streptomycin. A431 was obtained from the American Type Culture Collection (Rockville, MD). The cells were fed on alternate days with minimum essential medium (MEM) containing 10% FBS. The cells were maintained at 37°C under air plus 5% CO_2 conditions. All experiments using the cell lines were performed on a single clone.

Adenovirus vector infection of lung cancer cells

H1437 and A431 cells were plated at a density of 1×10^5 cells·$well^{-1}$ in 6-well plates (Costar, Cambridge, MA, USA), allowed to attach for 12 h and then nonadherent cells removed by gentle washing with phosphate-buffered saline (PBS). One day after plating, the total cell number·$well^{-1}$ was approximately 1×10^5–3×10^5 (n=5). Wells were then randomly assigned to one of six experimental groups: MOI of 1, 10, 10^2, 10^3, or 10^4 or vehicle control (RPMI or MEM+0.4% FBS). The MOI was determined in HEK293 cells [15, 17]. The Ad vector was added to the wells in 1.5 mL of RPMI or MEM containing 0.4% FBS, the cells exposed to it for 1 h at 37°C under air plus 5% CO_2 conditions and then washed with PBS and fed with fresh RPMI or MEM containing 0.4% FBS. The control cultures were exposed to 1.5 mL vehicle (RPMI or MEM +0.4% FBS).

Assessment of efficiency of gene transfer to lung cancer cells

One day after infection, the transduction efficiency was quantified as the percentage of *lacZ*-expressing cells *via* X-Gal staining [15–18]. In brief, cultured cells were washed with PBS, fixed for 8 min in 0.5% glutaraldehyde in PBS and then washed with PBS containing 1mM $MgCl_2$ and left for 15 min at 4°C. The cells were overlaid with freshly prepared histochemical reaction mixture containing 1 mg·mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 5mM potassium ferricyanide, 5mM potassium ferrocyanide and 1mM $MgCl_2$ in PBS for 4 h at 37°C. To assess the duration of transgene expression after vector-infection, X-Gal staining was performed daily up to 7 days after Ad vector- or vehicle-exposure. In all experiments, at least three wells from each group were stained for *lacZ* gene expression and >500 cells·$well^{-1}$ counted to determine the percentage of *lacZ*-expressing cells.

Cell growth and viability after adenovirus vector-infection

The cell proliferation rate was determined by counting the cells every other day for 7 days after Ad vector-infection. The cell numbers in three wells from each group were calculated using a standard haemacytometer following cell detachment with 0.1% trypsin plus 1 mM ethylenediamine-tetraacetic acid (EDTA) in PBS (trypsin–EDTA solution). Cell viability was assessed by means of their ability to exclude trypan blue. The doubling time (t_D) of the cell population was calculated according to the following formula: $t_D=(t - t_0) \times \log 2/(\log N - \log N_0)$ where N_0 is the number of cells at day zero (t_0) during the log phase of the growth curve and N is the number of cells at day t during the log phase near the plateau of the curve.

Detection of apoptosis after adenovirus vector infection of lung cancer cells

Deoxyribonucleic acid fragmentation assay. The deoxyribonucleic acid (DNA) fragmentation assay was performed following the method described by Tilly and Hsueh [20]. DNA was extracted from H1437 cells after exposure to Ad vectors for 1 h at MOIs of 1, 10^2 or 10^4 or vehicle. End-labelling 3′of DNA was performed according to the method of Roychoudhury *et al.* [21] using radiolabelled dideoxynucleotide (α-^{32}P-dideoxyadenosine triphosphate (ddATP) (3,000 Ci·$mmol^{-1}$), Amersham, Arlington Heights, IL, USA). The volume of the DNA sample in sterile water was adjusted to 29 μL. To these samples were added 10 μL 5 × reaction buffer (1 M potassium acetate, 1.25 mg·mL^{-1} bovine serum albumin; pH 6.6), 5 μL 25 mM $CoCl_2$, 5 μL α-^{32}P-ddATP (50 μCi) and 1 μL (25 IU) terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim, Tokyo, Japan); the mixture was incubated for 1 h at 37°C. The reaction was terminated by the addition of 5 μL 0.25 M EDTA. Labelled DNA was separated from unincorporated radionucleotide by the addition of 0.2 volumes of 10 M ammonium acetate, 3 volumes of 100% ethanol and 1 μL 20 μg·μL^{-1} glycogen for 60 min at -70°C. The nucleic acid was collected by centrifugation, washed with 0.25 mL 80% ethanol and resuspended in 20 μL 10 mM tris (hydroxymethyl) aminomethane (Tris)–HCl, 1 mM EDTA (pH 7.5). DNA samples were loaded on a 2% agarose gel and separated by electrophoresis for 3.5 h at 50–60 V using 0.04 M Tris–HCl, 0.02 M NaOH, 1 mM EDTA (pH 7.8) as running buffer. The gel was dried using a slab gel drier (Model SE 1160; Hoefer Scientific Instruments, San Francisco, CA, USA) for 2 h without heat, and exposed to Fuji radiography film (Fuji Photo Film Co., Tokyo, Japan) for 2–48 h at -70°C.

Morphological characteristics of cells after adenovirus vector-infection. Morphological characterization of cells was performed *via* transmission electron microscopy. One day after seeding H1437 cells (10^6) in two 60 mm dishes; cells in one dish were exposed to Ad vector

(MOI of 10^4, 1 h) and in the other dish to vehicle (1 h). At 24 h after Ad vector exposure, the detached cells in the culture medium of the Ad vector-exposed dish were harvested by centrifugation at $200 \times g$. The adherent cells in the vehicle-exposed dish were collected using 1 mM EDTA. The harvested cells were immediately fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and then postfixed using 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4°C. After alcohol-dehydration, the cells were embedded in Epon Resin (Sigma Co., St Louis, MO, USA) according to the standard method [22, 23], and sectioned using an LKB-Huxley ultramicrotome (LKB Produkter, Bromma, Sweden) to a final thickness of 60–90 μm. Sections were stained with 2% uranyl acetate and 2% lead acetate, and then examined using a Hitachi H-7100 transmission electron microscope (Hitachi, Tokyo, Japan).

Quantification of apoptosis in culture dish. To determine the ratio of apoptotic cells to total cells in the culture dish, modified TdT-mediated deoxyuridine triphosphate (dUTP)–biotin nick end-labelling (TUNEL) was performed using Apoptag® (Oncor Co. Gaithersburg, MD, USA) [12, 23]. Lung cancer cells (H1437 and A431) were plated at a density of 3×10^5 cells·well^{-1} in 6-well culture plates (Costar). One day after seeding, nine wells were exposed to each concentration of Ad vectors, at MOIs of 1, 10^2 and 10^4, or vehicle for 1 hr. At 24, 48 and 72 h after Ad vector-infection, the cells were harvested using trypsin–EDTA solution and stained by means of a modified TUNEL method. In brief, the cells were harvested from the culture plate using trypsin–EDTA and fixed with 2% paraformaldehyde for 15 min on ice. The cells were washed with PBS and collected by centrifugation (5 min at $200 \times g$). The cells were centrifuged as before, the supernatant removed and the cells resuspended in 32 μL equilibration buffer. After centrifugation for 5 min at $200 \times g$, the supernatant was removed and the cells incubated with 8 μL TdT and 19 μL reaction buffer for 1 h at 37°C. The reaction was terminated by the addition of 20 μL stop buffer and 680 μL distilled water. After centrifugation, the supernatant was removed and the pellet resuspended in 20 μL anti-digoxigenin–fluorescein and 38 μL blocking solution and then incubated for 30 min at 20°C. Ten microlitres of 0.1% Triton X-100 in PBS was added to the cell suspension. After centrifugation, the supernatant was removed, the cells resuspended in 1 μg propidium iodide (PI) (Sigma Co.) and 10 μg ribonuclease (RNase) (Boehringer Mannheim Co., Tokyo, Japan) in 200 μL PBS for 15 min and then washed with 200 μL PBS. The anti-digoxigenin–fluorescein-positive (apoptotic cells) were counted using a haemacytometer and a fluorescence microscope (EPI-FL3; Nikon Microphot Tokyo, Japan). Total cell numbers were determined by counting PI-stained cells. The ratio of apoptotic to total cells in each sample (≥500 cells counted in each sample) was calculated.

Measurement of cell cycle of lung cancer cells after adenovirus vector-infection

For cell cycle experiments, H1437 cells were plated at a density of 1×10^6 cells·dish^{-1} in 10 cm culture dishes (Costar). One day after seeding, the cells were exposed to Ad vectors, at MOIs of 1, 10^2, 10^4 or vehicle for 1 h. At 24, 48 and 72 h after Ad vector-infection, the cells were harvested using 1mM EDTA solution. They were then washed twice in PBS and the pellets fixed in 80% ethanol for 1 hr at 4°C. Fixed cells were centrifuged, resuspended in PBS, recentrifuged and resuspended in a solution containing 750 μg·mL^{-1} RNase A and 50 μg·mL^{-1} PI in PBS. Samples stained with PI were analysed for DNA content using a FACScan (Becton Dickinson, San Jose, CA, USA). Cell cycle distribution was determined using CELL FIT software (San Jose, CA, USA) on a HP340 Series 9000 Workstation. Dead cells were gated out using pulse processing.

Effect of inactivation of adenovirus vector on cell kinetics in lung cancer cells

Ad vector genes were inactivated by incubation for 1 h at 60°C. The effects of inactivation of Ad vector on cell proliferation were tested in lung cancer cell lines. One day after plating, cells were exposed to inactivated Ad vectors and untreated Ad vector at a MOI of 10^4 or vehicle (RPMI or MEM+0.4% FBS) for 1 h. Cell proliferation was determined by counting the cells every other day until 7 days after Ad exposure.

Statistical analysis

Data are presented as mean±SD. Statistical analysis was performed using analysis of variance (ANOVA) with Fisher's protected least significant difference method or Student's t-test using Stat View 4.0 (Abacus Concepts, Inc., Berkeley, CA, USA). A p-value <0.05 was considered statistically significant.

Results

Kinetics of the gene transfer efficiency of the adenovirus vector in cancer cells

Although the transduction efficiency of the Ad vector was slightly better in H1437 cells than in A431 cells at MOIs of 10, 10^3 and 10^4, there was a dose-dependent relationship between efficiency of *lacZ* gene transfer to cancer cells and vector MOI in each cell line (fig. 1a). The changes in the percentage of *lacZ*-transduced cells in culture with time are depicted in figure 1b. In each lung cancer cell line, the transduction efficiency of the Ad vector decreased with time after Ad vector-infection (fig. 1b).

Growth curves of lung cancer cells with or without adenovirus vector-infection

The kinetics of cell proliferation in H1437 cells after exposure to the Ad vector or vehicle was similar to that in A431 cells. The growth curves of Ad vector-infected cells were shifted to the right in a MOI-dependent manner (fig. 2). While the accelerated phase of the growth curve was initiated one day after vehicle-exposure, log phase growth

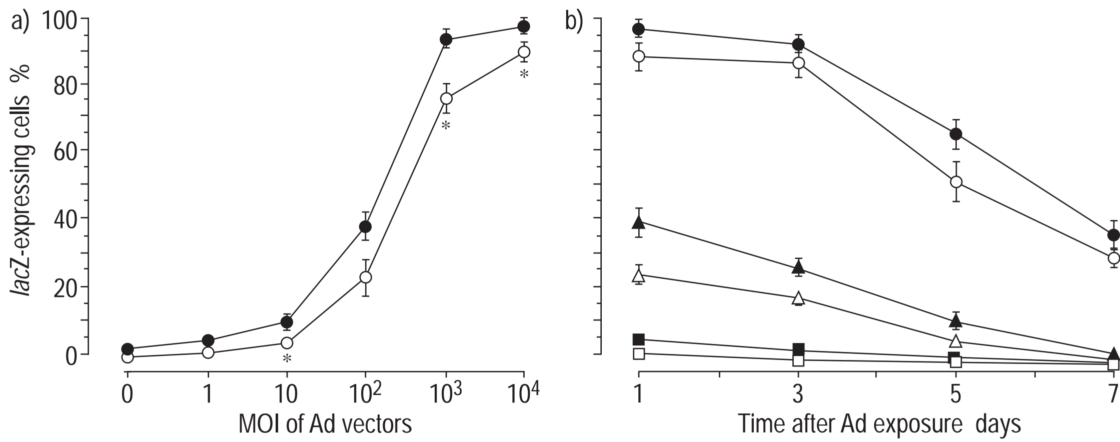

Fig. 1. – The efficiency of: a) hAd5-CMV-*lacZ*-mediated gene (*lacZ*) transfer in H1437 (●) and A431 cells (○) 1 day after infection (*: p<0.05 *versus* H1437); and b) transduction of Adenovirus (Ad) vector with time after Ad vector infection of H1437 (■: multiplicity of infection (MOI) 1; ▲: MOI 10^2; ●: MOI 10^4) and A431 cells (□: MOI 1; △: MOI 10^2; ○: MOI 10^4).

started 3 days after Ad vector-infection in both cell lines. The delayed onset of log phase growth was caused by the longer t_D of Ad-infected cells compared to that of vehicle-exposed cells (table 1). Further, the viability of the cells, as determined by trypan blue exclusion, was lower in vector-exposed cells than in vehicle-exposed cells (fig. 3). The lower viability of the Ad vector-infected cells compared with vehicle-exposed cells lasted ≥48 h after Ad vector-infection.

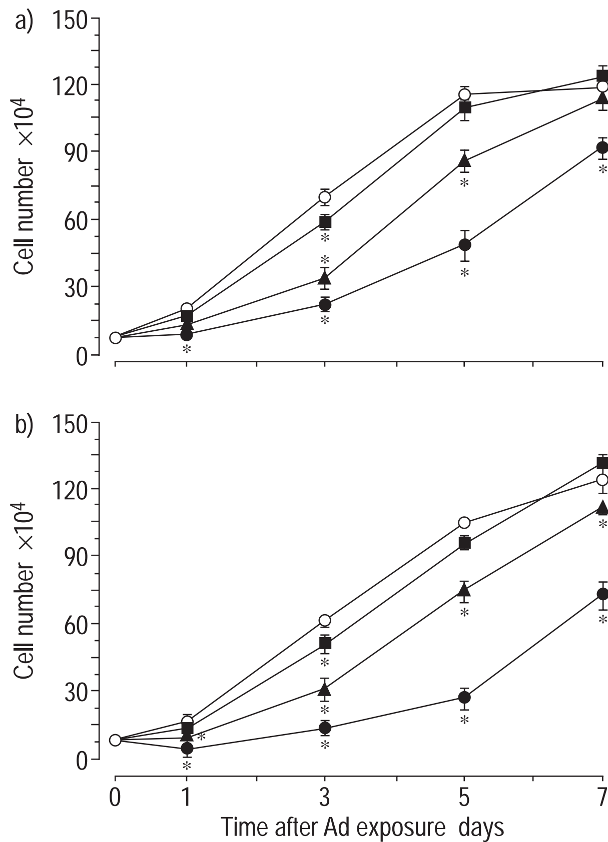

Fig. 2. – Kinetics of cell proliferation after exposure to hAd5-CMV-*lacZ* (■: multiplicity of infection) MOI 1; ▲: MOI 10^2; ●: MOI 10^4) or vehicle (○) in: a) H1437; and b) A431 cells. Values represent mean±SD (n=3). Ad: adenovirus. *: p<0.05 *versus* vehicle.

Detection of apoptosis in adenovirus vector-infected cells

DNA integrity analysis utilizing agarose gel electrophoresis of H1437 cells with or without Ad vector-exposure is shown in figure 4. DNA fragmentation was not obvious in the DNA extracted from vehicle-exposed cells. On the other hand, DNA fragmentation was clearly present in DNA extracted from cells exposed to Ad vector at a higher MOI. Transmission electron micrographs of vehicle-exposed H1437 cells and Ad vector-exposed H1437 cells are shown in figure 5. Compared with vehicle-exposed cells, Ad vector-infected cells (MOI of 10^4) revealed the features of apoptosis, such as cell shrinkage, nuclear condensation and fragmentation, and apoptotic body formation. Morphological features of apoptosis were found in cells infected with Ad vector at MOIs of 10^4 and 10^2. However, the percentage of apoptotic cells was lower in cells infected with Ad vector at MOIs of 1 and 10^2 than in those infected at a MOI of 10^4. The percentage of apoptotic cells was quantified by means of DNA strand breaks detected using TUNEL (fig. 6). The ratio of apoptotic (TUNEL-positive) cells to total cells increased with increasing vector MOI. However, the number of apoptotic cells after Ad vector-infection decreased with time in culture.

Table 1. – Doubling time (t_D) of lung cancer cell lines with and without adenovirus (Ad) vector-exposure

	t_D days	
Vector	H1437	A431
Vehicle	1.55±0.06	1.55±0.07
Ad, MOI 1	1.53±0.07	1.62±0.12
Ad, MOI 10^2	1.70±0.13	1.75±0.14
Ad, MOI 10^4	1.81±0.11	1.88±0.16
UV-inactivated Ad, MOI 10	1.56±0.06	1.56±0.10

Values are presented as mean±SD. t_D was calculated according to the formula given in *Methods*. MOI: multiplicity of infection; UV-inactivated Ad: ultraviolet plus psolaren-inactivated Ad vectors.

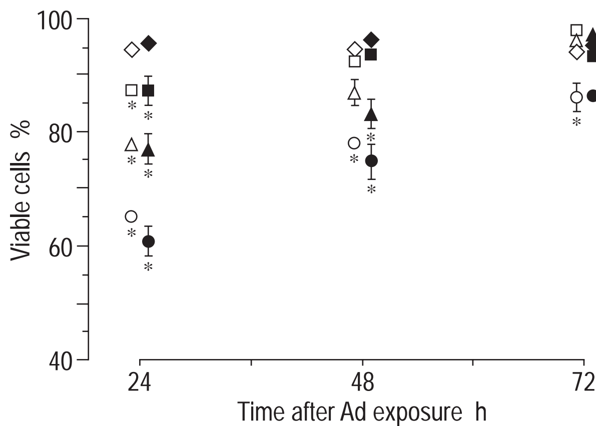

Fig. 3. – Viability of cells as determined by trypan blue exclusion as a function of time after vector or vehicle (H1437: ◆; A431:◇)-exposure of H1437 (■: multiplicity of infectoin (MOI) 1; ▲: MOI 10^2; ●: MOI 10^4) and A431 cells (□: MOI 1; △: MOI 10^2; ○: MOI 10^4). Values are presented as mean±SD (n=3). Ad: adenovirus. *: $p<0.05$ *versus* vehicle.

Effect of adenovirus vector-infection on cell cycle in lung cancer cells

The percentage of cells in the DNA synthetic (S) phase after Ad vector-exposure was lower than that after vehicle-exposure. There was a decrease in the number of cells in the S phase 24–48 h after Ad vector-exposure, accompanied by an increase in the percentage of cells in the quiescent (G_2)/mitosis (M) phase (fig. 7). There was no difference in the percentage of cells in the interphase (G_1) phase between vector- and vehicle-exposed cells. In cells infected with Ad vector at a MOI of 10^2, a reduction in the number of cells in the S phase 24–48 h after Ad vector-exposure, accompanied by an increase in the percentage of cells in the G_2/M phase, was also observed. However, this alteration of the cell cycle was not observed in the cells infected with Ad vector at a MOI of 1.

*Effect of heat-inactivation of genes of Ad-CMV-*lacZ *on the growth kinetics of lung cancer cells*

The effect of heat-inactivation of genes of hAd5-CMV-*lacZ* on the proliferation of cancer cells is shown in figure 8. There was no difference in growth curves between heat-inactivated Ad vector (MOI=10^4)-exposed cells and vehicle-exposed cells. The doubling times of cells after exposure to Ad vector or heat-inactivated Ad vector are presented in table 1. Inactivation of vector genes almost completely abrogated the Ad vector effects on cell kinetics in lung cancer cells. In addition, there was no difference in growth curves between heat-inactivated Ad vector (MOI=10^2)-exposed cells and vehicle-exposed cells, or between heat-inactivated Ad vector (MOI 1)-exposed cells and vehicle-exposed cells

Discussion

The present study demonstrated that E1-deleted Ad vector-infection inhibited proliferation of cultured lung carcinoma cells. The retarded growth after Ad vector-infection was characterized by both the delayed onset of log phase growth and slower proliferation during the

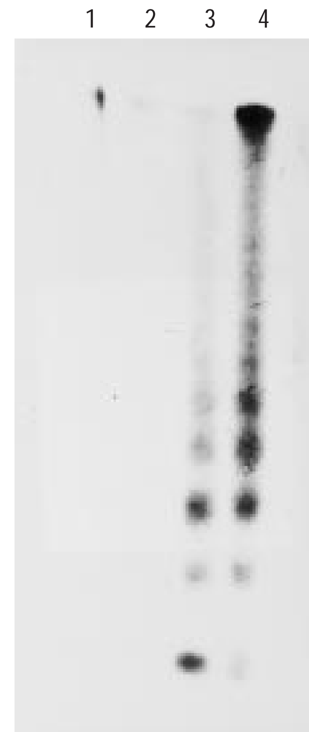

Fig. 4. – Deoxyribonucleic acid (DNA) integrity analysis ("DNA ladder") utilizing agarose gel electrophoretic separation of α-^{32}P-dideoxyadenosine triphosphate 3′ end-labelled DNA samples from H1437 cells exposed to vector (hAd5-CMV-*lacZ*, lane 2: multiplicity of infection (MOI) 1; lane 3: MOI 10^2; lane 4: MOI 10^4) or vehicle (lane 1). Each sample consisted of cells harvested from dishes 24 h after exposure to vector or vehicle. The material in each lane was extracted from the same amount of DNA (200 ng).

accelerated growth phase, as indicated by the longer *t*D. It can be assumed that the increased apoptotic death of vector-infected cells was associated with the delayed onset of log phase growth. In fact, Ad vector infection with a high MOI reduced the viability of lung cancer cell lines. The morphology of the cells after Ad vector-exposure revealed apoptotic features including cell shrinkage and nuclear condensation and fragmentation [12, 24]. Internucleosomal DNA fragmentation was increased in Ad vector-infected cells in a vector titre-dependent manner. This finding was confirmed by the increased formation of DNA strand breaks in Ad vector-exposed cells, as determined by the TUNEL assay. However, whereas these findings were clear-cut in cells infected with a high MOI, they were less obvious in those with a low MOI, suggesting that a high MOI of recombinant-defective Ad vector can induce apoptosis in lung cancer cells lines.

Many Ad early genes, including E1a and E3, are known to induce apoptosis and/or inhibit cytolysis in infected cells [25–27]; these genes have been deleted from Ad vectors utilized for cancer gene therapy. It has not yet been determined whether or not other Ad genes present in Ad vectors can induce or inhibit the apoptotic cell death of lung cancer cells. Further since lung cancer cells can be immortalized by oncogenes involved in induction and/or inhibition of apoptosis, the mechanism of Ad vector-induced apoptosis in lung cancer cells may not be straightforward. Several lines of evidence suggest that Ad gene products increase p53 levels and induce p53-dependent

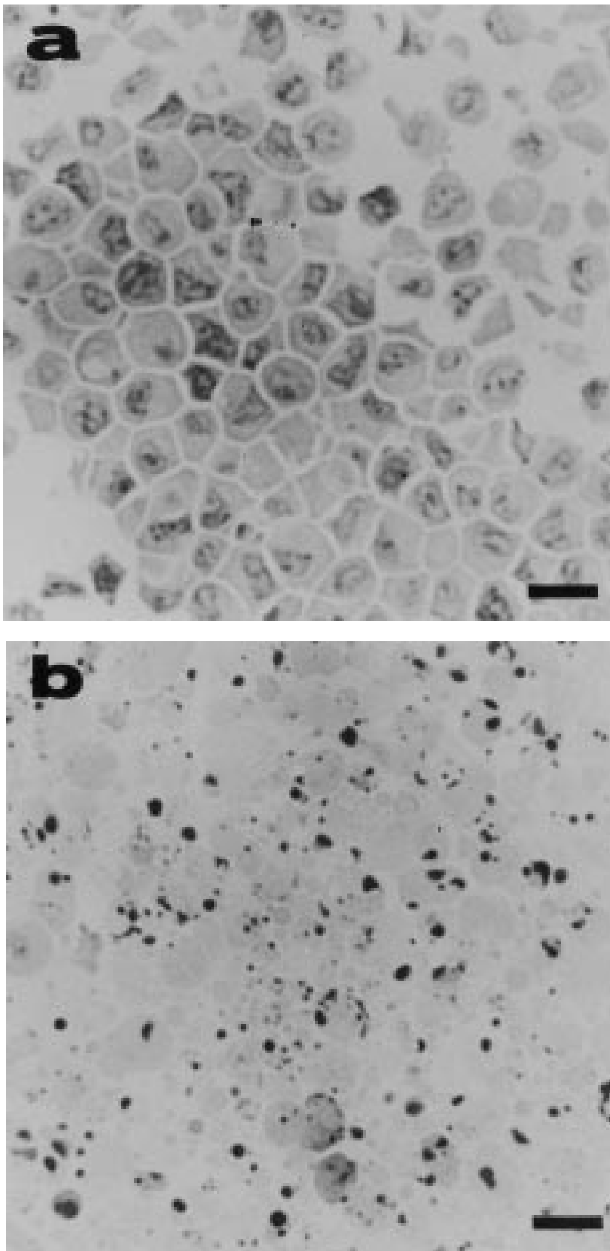

Fig. 5. – Transmission electron micrographs of: a) adherent vehicle-exposed H1437 cells harvested from the culture dish using ethylene-diamine tetraacetic acid solution; and b) detached H1437 cells exposed to adenovirus vector (hAd5-CMV-l*ac*Z) at a multiplicity of infection (MOI) of 10^4. Internal scale bar = 20 µm.

apoptosis [26, 27]. However, KATAYOSE *et al.* [28] have reported that E1a/E1b region-deleted Ad vectors do not upregulate *p53* expression in certain cells. SUBRAMANIAN *et al.* [29] have found that E1b-partially deleted 19 k mutant adenoviruses induce apoptosis in primary mouse kidney cells prepared from *p53* (+/+) as well as *p53* (-/-) mice. These observations suggest that *p53* expression may not play a major role in Ad vector-induced apoptosis in lung cancer cells. In the present study, the growth-inhibition caused by Ad vector-infection was investigated in two cancer cell lines and found to be similar, indicating that Ad genes rather than oncogenes present in the cancer cells are involved in the mechanism of apoptosis. However, the greater amount of vector protein present at a higher MOI seems to be harmful to cells in culture. Thus, whether vector-induced apoptosis was due to vector gene expression or vector proteins was examined. The results demonstrated that heat-inactivation of vector genes almost entirely abolished the effect, suggesting that apoptosis reflects vector gene expression rather than the toxicity of vector proteins. BROUGH *et al.* [30] have demonstrated that E1- and E4-deleted Ad vector achieves persistent transgene expression in the liver and lungs of mice [30]. In addition, it has recently been reported that adenovirus type 5 E4 open reading frame 4 induces apoptosis in transformed cells *in vitro* [31, 32]. Taken together, these results indicate that Ad E4 genes present in the current E1- and E3-deleted Ad vectors may be responsible for the vector-induced apoptosis of lung cancer cells.

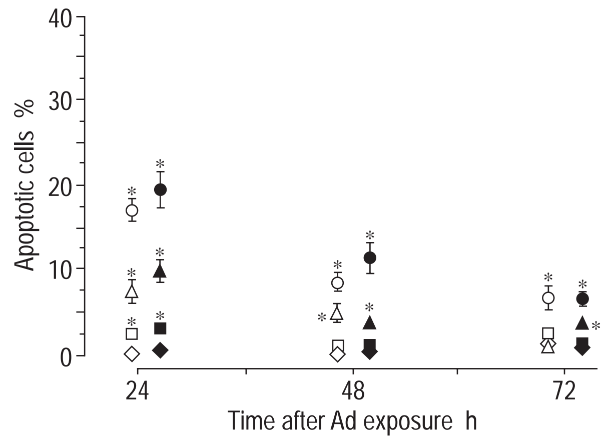

Fig. 6. – Effect of adenovirus (Ad) vector (hAd5-CMV-*lacZ*)-infection on induction of apoptosis in H1437 (■: multiplicity of infection (MOI) 1; ▲: MOI 10^2; ●: MOI 10^4) and A431 cells (□: MOI 1; △: MOI 10^2; ○: MOI 10^4). Apoptosis was measured using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick end-labelling. Values are presented as mean±SD (n=3). *: $p<0.05$ *versus* vehicle.

Cell cycle distribution was examined after Ad vector-infection of cancer cells by means of FACScan analysis after staining with PI. Although the percentage of cells in

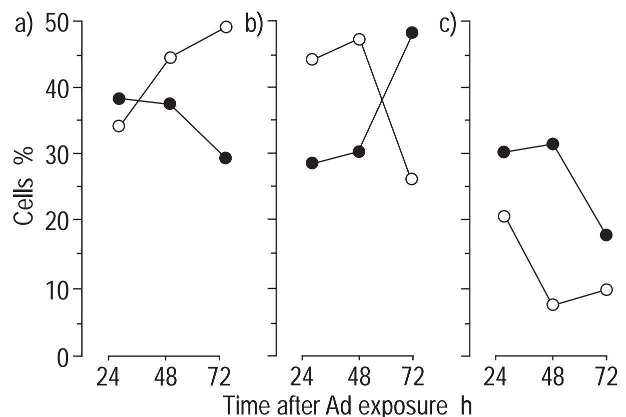

Fig. 7. – Effect of adenovirus (Ad) vector (hAd5-CMV-*lacZ*)-infection on the cell cycle in lung cancer cells. The percentages of cells in: a) the interphase (G_1); b) the deoxyribonucleic acid synthetic (S); and c) the quiescent (G_2)/mitosis (M) phases of the cell cycle are presented as a function of time after Ad vector-infection. ○: vehicle-exposed cells; ●: Ad vector (MOI 10^4)-exposed cells.

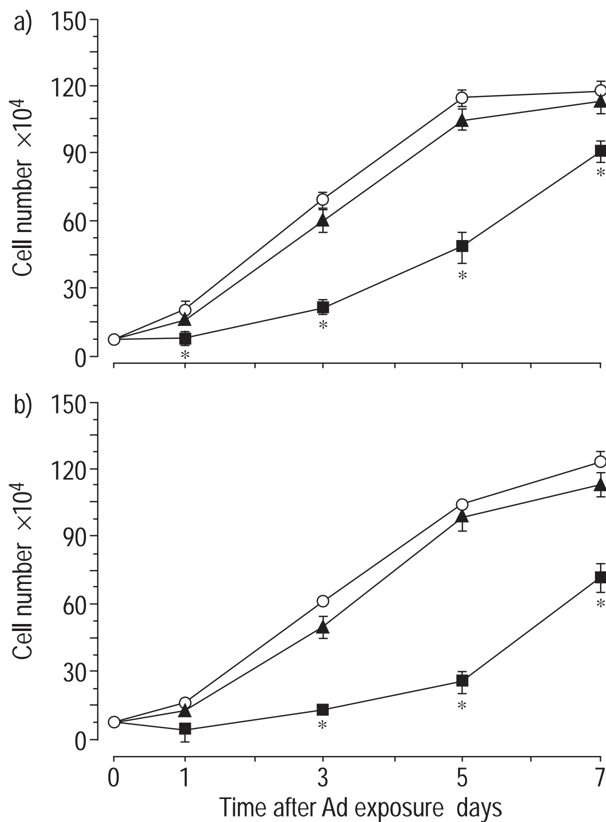

Fig. 8. – Effect of heat-inactivation of adenovirus (Ad) vector (○: vehicle; ▲: ultraviolet-inactivated Ad, multiplicity of infection (MOI) 10^4; ■: Ad, MOI 10^4) on proliferation of a) H1437; and b) A431 cells. Values are presented as mean±SD (n=3). *: $p<0.05$ *versus* vehicle.

G_1 was not different between vector- and vehicle-exposed cells, a decrease in the number of cells in the S phase accompanied by an increase in the percentage of cells in the G_2/M phase was found in Ad vector-exposed cells. Thus, the longer *t*D of vector-exposed cells may be explained by the lower percentage of cells recruited into the S phase after Ad vector-exposure. Although apoptosis is closely associated with G_1 arrest in several cell types [33, 34], the present results indicate that apoptosis caused by Ad vector is not always related to G_1 arrest. Since there are two points, the transitions between G_1/S and G_2/M, at which DNA damage is monitored, the accumulation of cells in the G_2/M transition phase may be associated with Ad vector-induced apoptosis in cancer cells.

In the current study, the efficiency of Ad vector-mediated gene transfer to cancer cells confirmed that previous reports [1–3, 10]. Replication-incompetent Ads are efficient vectors, having a high efficiency of gene transfer, compared with other vector systems, in culture. However, the transduction efficiency of the Ad vector was slightly different in the two cancer cell lines investigated. The present study thus, suggests that adenocarcinoma may be better transduced by Ad vectors than is squamous cell carcinoma. Although replication-defective Ad vectors are known to infect various types of cells, the efficiency of Ad-mediated gene transfer is not the same in different types of cells [15–17, 35]. Further studies are needed to elucidate the relationship between lung cancer cell type and the gene transfer efficiency of Ad vectors.

In summary, adenovirus vector-mediated gene transfer to lung cancer cells was highly efficient in a multiplicity of infection-dependent fashion. Adenovirus vectors at higher multiplicities induced apoptotic cell death in adenovirus vector-infected cancer cells and subsequently reduced the transduction efficiency of adenovirus vectors with time. Since a higher multiplicity of infection of adenovirus vectors may be necessary to transduce foreign gene into target cells *in vivo*, adenovirus vector-induced apoptosis may reduce transgene expression in lung cancers. These effects of adenovirus vectors on cell kinetics are disadvantageous to the efficacy of gene therapy of lung carcinoma. Elimination of these effects of adenovirus vectors on lung cancer cells may be important in improving the efficacy of adenovirus-mediated gene therapy.

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