Eur Respir J 2002; 20: 703–709
DOI: 10.1183/09031936.02.00294502
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European Respiratory Journal
ISSN 0903-1936

Adenovirus-mediated E2F-1 gene transfer in nonsmall-cell lung cancer induces cell growth arrest and apoptosis

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ABSTRACT: Since overexpression of E2F-1 has been shown to induce apoptosis, the ability of adenovirus-mediated transfer of E2F-1 to inhibit tumour growth in nonsmall-cell lung cancer cell lines was investigated.

Three cell lines with various genomic status were infected with AdE2F. Cell proliferation and viability were determined by trypan blue exclusion. Apoptosis induction was assessed by flow cytometry and poly-adenosine diphosphate-ribose-polymerase cleavage assay. *In vivo*, the effect of E2F-1 on tumour growth was determined in severe combined immunodeficiency (SCID) mice.

The current experiments showed that overexpression of E2F-1 suppressed tumour cell growth. The population of apoptotic cells was dramatically increased 96 h after infection with AdE2F. Inhibition of cell growth and induction of apoptosis was not dependent on genomic status. Moreover, treatment of implanted tumours in SCID mice with AdE2F inhibited tumour growth.

These data suggest that adenovirus-mediated E2F-1 gene therapy may be effective in the treatment of nonsmall-cell lung cancer.
Eur Respir J 2002; 20: 703–709.

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Keywords: E2F-1, gene therapy, nonsmall-cell lung carcinoma, p53

Received: November 10 2001
Accepted after revision: February 10 2002

Despite the development of new chemotherapeutic agents and treatment protocols, and considerable progress in surgical and radiotherapy techniques, the low survival rate of patients with lung cancer has hardly changed. Consequently, new therapeutic strategies are needed. A promising approach for a molecular therapeutic strategy of nonsmall-cell lung cancer (NCSLC) is the replacement of inactivated tumour suppressor genes. Candidates for gene replacement of inactivated tumour suppressor genes are p53 and p16, which are inactivated in >50% of human NSCLC [1, 2]. First, clinical trials to replace the p53 function in patients with NSCLC are encouraging [3, 4]. All investigators showed that intratumoural injection of retroviral or adenoviral vectors expressing wild-type p53 complementary deoxyribonucleic acid (cDNA) is well tolerated. Furthermore, some patients showed evidence of tumour regression. Replacement of the p16 gene is also an alternative strategy to trigger the apoptosis pathway. *In vitro* investigations have shown that the transfer of p16 induced apoptosis in NSCLC cell lines that did not express p16 [5], but this required the expression of wild type p53 [6]. However, suppression of cancer cell proliferation and tumour growth after transfer of p53 or p16 wild-type cDNAs was only observed in cancer cells without expression of intact p53 or p16, respectively [7, 8]. Therefore, the current authors' interest was focused on alternative genes that induce apoptosis and growth arrest independent of the genomic status of the tumour.

The E2F-1 gene is one of the best-characterised members of the E2F transcription factor family. It was identified by its binding affinity to the Retinoblastoma (Rb) tumour suppressor gene product [9]. For transition from G_0/G_1 to S phase, phosphorylation of retinoblastoma protein (pRb) by G_1 cyclin/cyclin-dependent kinase (cdk) complexes results in release of active E2F-1 protein [10]. Active E2F-1, through its function as transcription activator directs the timely expression of cell cycle-controlling genes, whose products are implicated in G_1- and S-phase progression [11]. Several studies have shown that changes in the expression level of E2F-1 protein can lead to dysregulation of the cell cycle. Overexpression of E2F-1 induces apoptosis and suppresses tumour growth *in vitro* and *in vivo* in different cancer cell lines [12–15]. In addition, mice lacking E2F-1 develop a broad and unusual range of tumours with high metastatic potential [16]. On the contrary, E2F-1 overexpression has also been shown to transform cells leading to induction of tumours in animal models [17]. These findings indicate that E2F-1 has a dual function in the regulation of the cell cycle, it is important in stimulating cellular proliferation but also in triggering of programmed cell death.

Therefore, in the current study, the effect of

adenovirus-mediated gene transfer of E2F-1 (AdE2F) on proliferation and apoptosis induction in NSCLC cell lines with different genomic status *in vitro* and on tumour growth *in vivo* were investigated. Treatment of NSCLC with a recombinant replication-deficient adenoviral vector (Ad) coding for E2F-1 caused inhibition of cell proliferation and induced apoptosis independent of the genomic status in NSCLC cell lines. Furthermore, gene transfer of E2F-1 suppressed tumour growth *in vivo*. These findings suggest that E2F-1 might be beneficial in gene therapy of NSCLC.

Material and methods

Cell lines and cell culture

The NSCLC cell lines H460, H1299, and H596 were purchased from American Type Culture Collection (Manassas, VA, USA). H1299 and H460 cell lines express pRb (pRb+) but not p16 (p16-). H460 expresses wild-type p53 (p53+) whereas p53 is deleted in H1299 (p53-). H596 is negative for pRb but positive for p16 (Rb-/p16+) and overexpresses mutated p53. All NSCLC cell lines and human embryonic kidney cells (293 cells) were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 or Dulbecco′s modified Eagle′s medium (DMEM), supplemented with 10% foetal calf serum and 2 mM glutamine at 37°C, 5% carbon dioxide (CO_2) in a humidified atmosphere. For experiments, cells were seeded, treated and analysed as indicated.

Recombinant adenovirus vector

The recombinant Ad vector AdE2F carries the cDNA of human E2F-1 under the control of the cytomegalovirus (CMV) promoter [18]. AdRSV-hAAT.2 carries the cDNA of the human alpha-1-antitrypsin driven by the Rous-Sarcoma-virus promoter. AdCMV.β-Gal expresses *Escherichia coli* β-galactosidase under the control of the CMV promoter. Vectors were propagated on 293 cells and purified, titred and stored at -80°C, as described previously [19]. Equal levels of infection in the different cell lines was determined by infecting the cells with AdCMV.β-Gal. Infection of cells was performed for 1.5 h at 37°C in serum-free medium. One day after infection, cells were fixed and stained for β-galactosidase activity as described previously [20].

Trypan blue staining

Cells were seeded at 1×10^6 cells in 10 cm dishes, allowed to adhere overnight and infected with Ad vectors at the indicated multiplicity of infection (MOI). The cells were harvested at the times indicated, stained with Trypan blue 0.4% and counted.

Western blotting

Cell extracts were prepared on ice with lysis buffer. Protein (10 $\mu g \cdot lane^{-1}$) was resolved by sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Hybond-C; Amersham Bioscience Corp., Piscataway, NJ, USA) by standard procedures. Primary monoclonal antibodies against E2F-1 (KH95/E2F) and PARP (C2-10) were from Pharmingen and the antibody against β-actin (N350) from Amersham Bioscience Corp. The biotinylated secondary antibody was from Vector Laboratories (Burlingame, CA, USA). Detection was performed with streptavidin coupled peroxidase and the ECL system (Amersham Bioscience Corp).

Assessment of apoptosis by fluorescence-activated cell sorter analysis

To evaluate the extent of apoptosis, cells were harvested 96 h after infection and stained with flourescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) using the Annexin V kit (Beckman Coulter, Brea, CA, USA) and analysed by fluorescence-activated cell sorter (FACS) (FACScan; Becton Dickinson Bioscience, San Jose, CA, USA).

To study cell cycle distributions, samples (5×10^5 cells) were fixed and permeabilised by the addition of 2 mL of ice-cold 70% ethanol for 1 h at 4°C. After washing, the cells were resuspended in 0.5 mL phosphate-buffered saline (PBS) containing 50 $mg \cdot mL^{-1}$ PI, pH 7.5. Following treatment with 10 μL of 10 $mg \cdot mL^{-1}$ ribonuclease (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min at room temperature in the dark, cells were analysed by flow cytometry.

In vivo *animal model*

H1299 cells (1×10^6 cells) were injected subcutaneous in the right flank of C.B17/BlnA-severe combined immunodeficiency (SCID)/SCID mice (SCID mice). After tumour formation adenoviral vector was administered intratumourally. Immediately before vector injection, the tumour size was measured in two dimensions and the volume (V) calculated according to the following equation:

$$V = a \times b^2/2 \quad (1)$$

with $a \geqslant b$. Only tumours measuring 10–40 mm^3 were included. The virus dose (30 MOI) administered was adjusted to the actual tumour size, assuming 1×10^6 $cells \cdot mm^{-3}$. For treatment of tumours, AdE2F was used. For control groups, PBS or AdRSV-hAAT.2 were inoculated, respectively. Tumour size was measured for 45 days. A nonparametric Friedman′s two-way analysis of variance test was used to test the statistical significance of the difference between tumour sizes of experimental group and control groups.

Results

E2F-1 is overexpressed in nonsmall-cell lung cancer cell lines infected with AdE2F

Firstly, the ability of the Ad vector to infect the NSCLC cell lines and to express exogenous E2F-1 was examined. A recombinant replication-deficient adenoviral vector (AdE2F) was used to deliver the cDNA of E2F-1 into NSCLC cells. Immunocytological investigations of all NSCLC cell lines showed that >70% of cells were positive for E2F-1 (data not shown). Furthermore, the Western Blot analysis from cell lysates of E2F-1-infected cells showed a similar level of E2F-1 in each cell line 24 h after infection (fig. 1b) This overexpression remained stable for at least 96 h. Mock-infected cells or cells infected with AdRSV.hAAT-2 control vector showed only a low level of endogenous E2F-1 expression (fig. 1a).

Overexpression of E2F-1 inhibits cell proliferation and induces apoptosis in nonsmall-cell lung cancer

To examine the biological effect of overexpression of exogenous E2F-1 on NSCLC cell lines, the growth of H460 and H1299 *in vitro* after infection with either AdE2F or AdRSV.hAAT-2, as a control vector, was investigated. For this purpose the total cell number was determined 24 h and 96 h after infection. There was no significant difference in the number of cells between mock treatment and infection with control vector 24 h after infection (fig. 2a). However, in cultures infected with AdE2F considerably fewer cells were observed when compared with controls. This growth-suppressive effect of AdE2F was even more prominent 96 h after infection in both cell lines (fig. 2b). At this time, ~70% of E2F-1-infected cells were detached from the culture dish. In addition, the number of cells was decreased to 20% of that of control virus-infected cultures. However, after 96 h the number of control virus-infected cells was also significantly lower than the number of mock-infected cells. The cause of this phenomenon was investigated in more detail and will be published elsewhere. Previous investigations have also shown that the adenovirus vector alone alters gene expression and the cell cycle of infected cells [21, 22]. Nevertheless, the current data indicate that treatment with AdE2F strongly inhibits the growth of both NSCLC cell lines, and that this effect is not dependent on the genomic status of p53 and p16.

Detachment of AdE2F-infected cells from the culture dish suggested the occurrence of apoptosis. To determine whether AdE2F treatment induces apoptosis in these NSCLC cells, infected cells were stained with Annexin V-FITC and analysed by FACS.

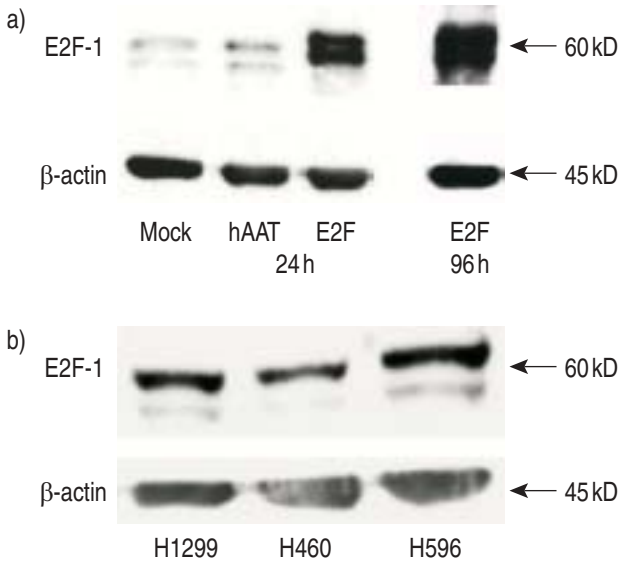

Fig. 1. – E2F-1 is overexpressed in nonsmall-cell lung cancer (NSCLC) cell lines infected with AdE2F. a) H1299 cells were mock infected (Mock) or infected with AdRSV-hAAT.2 (hAAT) or AdE2F (E2F) at a MOI of 25. Cells were harvested and prepared for Western blot analysis of E2F-1 24 h and 96 h after infection. b) NSCLC cell lines were infected with AdE2F (25 MOI for H1299, 75 MOI for H460, 200 MOI for H596). Cells were harvested and prepared for Western blot analysis 24 h after infection. β-Actin was used to control protein loading.

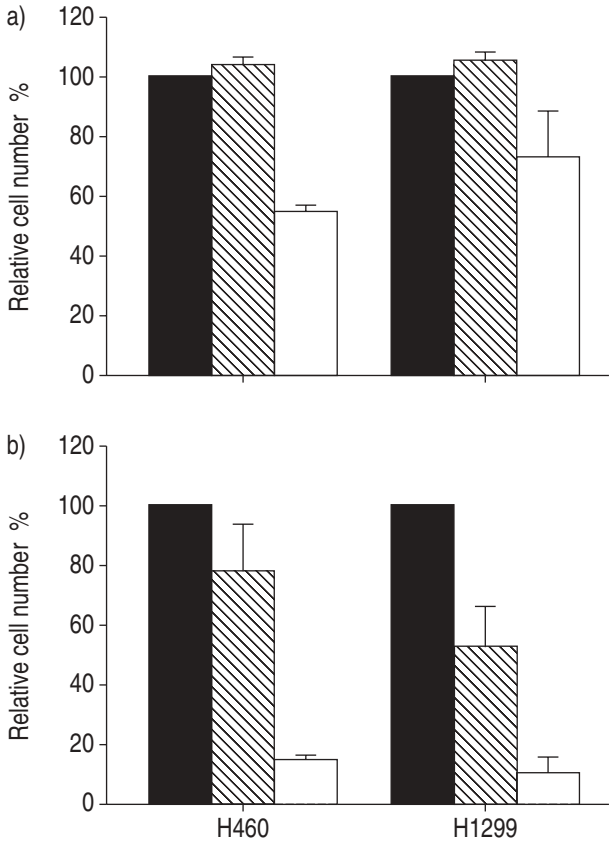

Fig. 2. – Infection of nonsmall-cell lung cancer cell lines with AdE2F inhibits cell proliferation. Cell lines were mock infected (■) or infected with AdRSV-hAAT.2 (▨) or AdE2F (□) (25 MOI for H1299, 75 MOI for H460). The total cell number was determined a) 24 h and b) 96 h after infection. The relative cell numbers are given with the mock treated samples set at 100%. The relative cell number is the mean±SD of three independent experiments.

Table 1. – Adenovirus-mediated overexpression of E2F-1 induces apoptosis

Cell line	Virus	Trypan blue staining[#]		FACS analysis[¶]
		24 h	96 h	96 h
H460	Mock	2.7±1.3	3.5±1.9	9.8±3.4
	Ad-hAAT.2	4.3±2.3	6.7±4.0	12.8±2.4
	AdE2F	4.6±0.7	49.7±13.7*	58.5±2.0*
H1299	Mock	1.7±0.3	2.4±0.5	1.9±0.2
	Ad-hAAT.2	2.1±0.6	4.7±2.8	6.8±0.4
	AdE2F	3.2±0.8	36.1±14.2*	44.1±2.5*

Data are presented as mean±SD. FACS: fluorescence-activated cell sorter. [#]: percentage of trypan blue positive cells; [¶]: percentage of Annexin V-positive cells; *: $p<0.05$.

No differences were observed between AdE2F-infected cells and controls 24 h after infection (data not shown). However, a dramatic increase in the fraction of Annexin V-positive cells 96 h after infection with AdE2F was observed (table 1). With the control vector, no increase or only a marginal increase in the number of apoptotic cells was seen. Trypan blue staining confirmed these results.

Furthermore, the degradation of poly-(adenosine diphosphate-ribose) polymerase (PARP), a 116 kD protein, that is a substrate for caspase-3 and is cleaved in apoptotic cells, was investigated. Western blot analysis showed that after 96 h PARP is completely cleaved in cells infected with AdE2F, whereas the control vector had no such effect (fig. 3). Although the extent of the observed effects on apoptosis induction differed in the two cell lines, the current data indicate that AdE2F did not only inhibit cell growth but also induced apoptosis and cell death in infected NSCLC cell lines, independent of p53 expression.

Effects of E2F-1 overexpression on cell cycle

To further investigate the mechanism of E2F-1-induced inhibition of cell growth and induction of apoptosis in NSCLC, cell-cycle distribution after infection with AdE2F was analysed. In H460 cells infected with AdE2F, an increase in the S and G_2/M

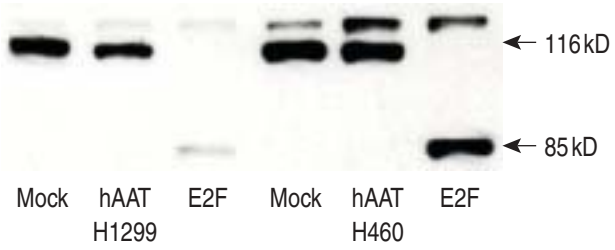

Fig. 3. – Adenovirus vector mediated transfer of E2F-1 induces cleavage of poly-(adenosine diphosphate-ribose) polymerase (PARP) in nonsmall-cell lung cancer cell lines. H1299 and H460 cells were mock infected (Mock) or infected with AdRSV-hAAT.2 (hAAT) or AdE2F (E2F). Cells were harvested 96 h after infection and prepared for Western blot analysis of PARP. The active form of PARP is shown as a 116 kD protein, whereas the cleaved inactive form of PARP appears as a 85 kD fragment.

Table 2. – Distribution of cell cycle phases 96 h after adenovirus vector infection

Cell line	Virus	G_0/G_1	S	G_2/M
H460	Mock	70.6±1.1	3.8±0.2	25.5±0.8
	Ad-hAAT.2	72.6±3.9	4.6±0.4	20.3±0.9
	AdE2F	45.8±0.9*	13.9±0.1*	39.3±0.9*
H1299	Mock	71.8±1.1	11.9±3.5	16.4±4.8
	Ad-hAAT.2	76.5±0.5	6.8±0.3	17.0±0.2
	AdE2F	7.3±0.7*	2.8±0.3*	90.1±0.5*

Data are presented as mean±SD. *: $p<0.05$.

phase populations were observed (table 2). Interestingly, in H1299 cells infected with AdE2F, there was only a dramatic increase in the cell population in G_2/M phase. The fraction of cells in G_0/G_1 phase was decreased in both cell lines. Mock-infected cells and cells infected with control virus showed similar results, with little alterations of normal cell cycle. These data suggest that E2F-1 overexpression is associated with changes of the cell cycle characterised by progression from G_0/G_1- to S-Phase and an arrest of cells in G_2. This phenomenon that E2F-1-infected cells are apparently unable to accomplish normal mitosis might account for the rapid growth inhibition after 24 h and subsequent apoptosis.

The extent of induction of apoptosis is dependent on the presence of retinoblastoma protein

Negative regulation of E2F-1 transcriptional activity and repression of E2F-responsive promoters is associated with pRb [11]. This led the current authors to investigate whether the presence or absence of endogenous pRb influences the ability of AdE2F to inhibit growth and induce apoptosis. The authors therefore compared the effect of AdE2F on H1299 (pRb+/p16-) and H596 (pRb-/p16+). Infection of H596 with AdE2F again resulted in fewer cells 24 h after infection, compared with controls, but no significant difference in the relative cell number between H1299 and H596 was observed (H1299: AdE2F 64.4±4.4%, H596: 56.5±5.3%; compared to mock-treated controls). However, as early as 24 h after infection ~70% of H596 cells were detached from the culture dish but no H1299 cells (data not shown). Furthermore, >30% of H596 cells stained positive for trypan blue (fig. 4). After 96 h all cells were detached and >70% were dead (data not shown). These findings indicate that a lack of pRb intensifies the induction of apoptosis leading to cell death.

Ad-mediated E2F-1 transfer in nonsmall-cell lung cancer suppresses tumour growth in an animal model

To study the effect of exogenous E2F-1 protein expression on tumour growth *in vivo*, the growth of H1299 tumours treated with AdE2F, AdRSV.hAAT-2 or PBS in SCID mice were compared. Tumour size was measured over 45 days until the animals had to be killed (fig. 5). A significant difference ($p<0.05$)

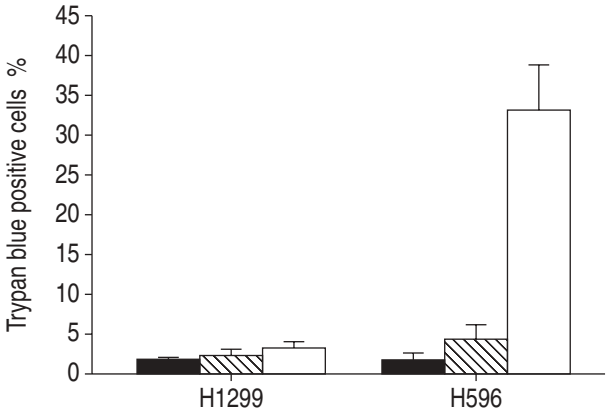

Fig. 4.–Induction of cell death is intensified in a retinoblastoma protein (pRb)-negative nonsmall-cell lung cancer cell line. Rb-positive H1299 (25 MOI) and Rb-negative H596 cells (200 MOI) were mock infected (■) or infected with AdRSV-hAAT.2 (▨) or AdE2F (□). Cells were harvested 24 h after infection and the proportion of dead cells was determined by staining with trypan blue. Columns represent the mean±SD of three independent experiments.

between the AdE2F treated group and control groups was observed 9 days following vector injection (AdE2F: 128.8±77.6 mm^3; PBS: 203.3±63.2 mm^3; AdRSV.hAAT-2: 208.3±65.0 mm^3). Tumour size in AdE2F treated animals was <50% of the size in control mice at day 9, and only 30% of controls at day 45 (AdE2F: 2241±647 mm^3; PBS: 8031±1441 mm^3; AdRSV.hAAT-2 7426±2130 mm^3). Furthermore, there was no significant difference between the two control groups. These data indicate that AdE2F suppresses tumour growth *in vivo*.

Discussion

Apoptosis, or programmed cell death is a highly conserved, innate mechanism of eukaryotic cells. This mechanism allows an organism to eliminate defective cells and retain tissue homeostasis. Deficiency of apoptosis is one substantial factor in tumourgenesis. Apoptosis regulating genes are a major target of cancerogeneous mutations. Consequently, the reconstitution of apoptotic mechanisms in tumour cells by gene delivery is a promising strategy for cancer therapy.

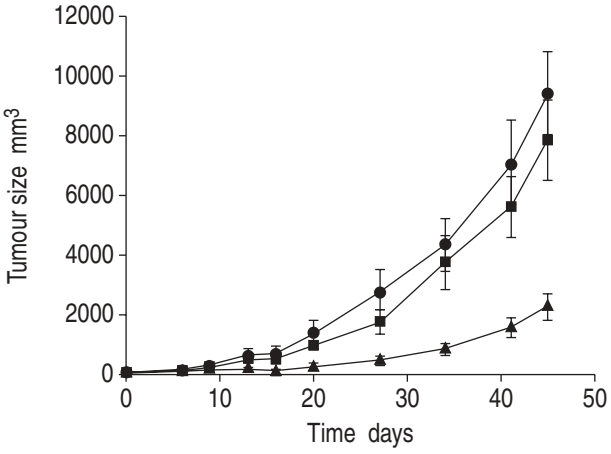

Fig. 5.–Suppression of tumour growth *in vivo* by infection with AdE2F. Subcutaneous tumours of H1299 cells in severe combined immunodeficiency (SCID) mice were treated with phosphate-buffered saline (●) (n=5), AdRSV-hAAT.2 (■) (n=8) or AdE2F (▲) (n=8) and tumour growth was measured over 45 days. Data present as mean±SE.

Gene therapy, by introducing wild-type p53, is a successful strategy in animal models of lung cancer and has been extended to human clinical studies [3]. The results were encouraging, but the therapeutic effect was limited to tumours with mutant p53. With only 50% of NSCLC carrying mutated p53, this approach is of limited applicability. Similarly, induction of apoptosis by transfection of p16 is also restricted to a subset of tumours because only 30% of NSCLC show no detectable p16 protein expression [2]. Because of these limitations of p53 and p16 gene therapy, due to the genomic status of tumours, the current authors used the transcription factor E2F-1 in this study, which has been shown to induce apoptosis in various cancer cell lines [12–15]. The current authors demonstrate that overexpression of E2F-1 by adenovirus-mediated gene transfer rapidly inhibits cell growth and induces apoptosis in NSCLC cell lines irrespective of the presence of functional p53 and p16. Furthermore, a single intratumoural injection of AdE2F in NSCLC tumour, efficiently suppresses tumour growth *in vivo*.

Earlier studies with E2F-1 have shown that this protein promotes cell cycle progression by activation of a series of genes that are necessary for transition from G_1- to S-phase [11]. Expression of E2F-1 can prevent cell cycle arrest of fibroblasts after serum deprivation [10] or overcomes a p16-mediated block in G_1 in these cells [23]. In this regard E2F-1 acts as a growth-stimulating factor. Contrary to this function of E2F-1, the current authors found an inhibition of cell proliferation *in vitro* as early as 24 h after infection. Because apoptosis was not observed until 96 h, this mechanism is not a likely cause for arrest of cell growth. However, an increased number of cells in S- and G_2/M phase have been observed following overexpression of E2F-1 [13–15]. It is therefore possible that E2F-1 induces cell cycle progression, but at the same time overexpression of E2F-1 also inhibits mitosis either directly or indirectly. Usually, during S-phase, E2F-1 binds to cyclinA/cdk2 and is phosphorylated by this cyclin-kinase complex [24]. This phosphorylation results in inhibition of E2F-1 DNA binding activity and is associated with cell entry into G_2/M [25]. A decreased activity of cyclin A/cdk2 will result in an increase of hypophosphorylated active E2F-1, which may lengthen S-phase or block cells in the S/G_2 stage [26]. Continuous overexpression of E2F-1 might disturb the balance between E2F-1 and cyclinA/cdk2 leading to the cell cycle effects observed by the current authors and others [13–15]. In summary, these data suggest that overexpression of E2F-1 induces a permanent S-phase entry with a block in the G_2/M phase. This deregulation of the cell cycle results in apoptosis, observed later in infected cell lines.

In the current study, it has been demonstrated

that induction of apoptosis is not dependent on p53 expression in tumour cells. These results are comparable to other studies with adenoviral-mediated gene transfer of E2F-1 in p53+ and p53- tumour cell lines [12–15]. Interestingly, in H460, considerably more apoptotic cells after AdE2F infection, in comparison with H1299, were found. Therefore, it might be that apoptosis induction is more effective in p53+ cells. ITOSHIMA *et al.* [27] have shown that overexpression of E2F-1 stabilises endogenous p53 protein expression and, in this way, renders cells more sensitive to apoptosis.

The current authors' investigations revealed an increase in cell death in a pRb-negative cell line infected with AdE2F compared with pRb-positive cell lines. Obviously the lack of pRb favours apoptosis of AdE2F infected cells. This supports the conclusion that induction of apoptosis is a result of permanent dysregulation of the cell cycle by E2F-1. It is well known that E2F-1 is negatively regulated by binding to hypophosphorylated pRb during G_1 phase of the cell cycle [11]. The loss of pRb will result in an increase of active E2F-1, which leads to cell cycle alterations such as uncontrolled S-phase entry or S/G_2 delay [28]. In Rb-negative H596 cells, inhibition of the overexpressed E2F-1 by pRb is absent. Therefore, in these cells the activity of E2F-1 is unrestricted and its effect more pronounced. This might explain the early induction of cell death 24 h after infection. The current observations are in line with other studies where the adenoviral transfer of E2F-1 in Rb-negative tumour cell lines enhanced cellular growth inhibition and induction of apoptosis [13, 14].

For tumour therapy in humans the effect of E2F-1 on noncancerous cells must always be considered. Several studies have shown that overexpression of E2F-1 also induced apoptosis in normal differentiated cells [29–32]. It is to be expected that overexpression of E2F-1 would probably also damage normal bronchial cells. Since NSCLC forms solid tumours, application of adenovirus carrying E2F-1 would have to be intratumourally, to reduce to a minimum the risk of serious toxicity to surrounding normal cells. Presently, studies to optimise the local administration of the vector are under way [33].

In conclusion, it has been demonstrated that E2F-1 is an efficient candidate gene for induction of growth suppression and apoptosis in nonsmall-cell lung cancer tumours *in vitro* and *in vivo*. Furthermore, the induction of these mechanisms by E2F-1 is not dependent on the p53 and p16 status of the tumour cells. These data indicate that the gene transfer of E2F-1 could be a successful strategy in lung cancer gene therapy, with the potential to treat a larger proportion of tumours than with the genes presently evaluated in clinical studies.

Acknowledgements. The authors would like to thank R. Crystal (Cornell University, New York, USA) and GeneVec Inc. for generously providing the adenoviral vectors; M. Gries and P. Pierschalek for their technical assistance; and W. Arnold for providing the SCID mice (University Medical Center Charité, Humboldt University of Berlin, Berlin, Germany).

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