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pRb-expressing adenovirus Ad5-*Rb* attenuates the p53-induced apoptosis in cervical cancer cell lines

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Abstract

The retinoblastoma protein (pRb), the gene product of the first reported tumour suppressor gene, is functionally inactivated by the E7 protein of high-risk human papillomavirus (HPV) found in most human cervical cancers. We have, in this study, constructed an adenoviral vector expressing wild-type pRb (Ad5-*Rb*) and used the constructed Ad5-*Rb* to transfect the osteosarcoma cell line Saos-2, and three cervical cancer cell lines HeLa, SiHa and C-33A. Our results showed that pRb caused G1 arrest in Saos-2 cells after transfection with Ad5-*Rb*. The number of colonies formed by the Ad5-*Rb*-transfected Saos-2 cells in soft agar was also found to be significantly lower ($P<0.05$) than those transfected with the adenoviral control expressing *Escherichia coli* β-galactosidase (Ad5-*LacZ*). The transfection of Ad5-*Rb* caused an increase in the population of SiHa and C-33A cells in the G1 phase from 53.0 and 52.9% to 72.4 and 64.3%, respectively, but not in the HeLa cells. However, Ad5-*Rb* did not show any inhibitory effect on the growth of SiHa, HeLa and C-33A cells, and inhibition of colony formation in soft agar was not observed either. In contrast, flow cytometric analysis showed that Ad5-*p53,* a p53-expressing adenovirus, induced apoptosis, i.e. the appearance of sub-G1 peak, in all three tested cervical cancer cell lines. Nevertheless, the Ad5-*p53*-induced apoptosis was partially inhibited when Ad5-*Rb* was added simultaneously. These findings suggested that pRb may not be a good candidate for cervical cancer gene therapy. Our data also showed that the use of full-length *pRb* in combination with *TP53* might not be a suitable strategy for cancer gene therapy.

Keywords: pRb; p53; Apoptosis; Adenoviral vector; Cervical cancer cell lines

1. Introduction

The human retinoblastoma gene (*Rb*) is the first reported tumour suppressor gene [1–3]. *Rb* encodes a 928 amino acid nuclear phosphoprotein commonly referred to as $p110^{Rb}$, pRb^{110} or pRb. pRb has an apparent molecular weight of 105–120 kDa depending on its state of phosphorylation and the phosphorylation status of pRb is tightly controlled throughout the mammalian cell cycle [4,5]. In the early G1 phase, pRb exists in a hypophosphorylated form. As cells proceed to late G1 phase, pRb is hyperphosphorylated by the cyclin-dependent kinases (cdk) and the level of phosphorylation of pRb increases during S phase and peaks in G2/M. In late M phase, pRb is dephosphorylated by a protein phosphatase [6]. When phosphorylated, pRb loses its ability to bind to the transcription factor E2F which allows the free E2F to stimulate the transcription of cellular genes including thymidine kinase, DNA polymerase α, dihydrofolate reductase, *jun*, *myb*, etc., and induce the G1/S transition [7].

Mutations of the *Rb* gene resulting in the loss of pRb function have been shown to be associated with human malignancies [8,9]. In the great majority of cervical cancers, pRb is functionally inactivated by the E7 protein of high-risk human papillomavirus (HPV) and the pRb inactivation leads to the disruption of the interaction between pRb and E2F [10,11]. Apart from pRb, the E7 protein can also bind to several pRb-related proteins including p107, p130 and p300, as well as cyclins A, D1 and E [12]. Study of cell-free extract prepared from cervical cancer cells with E7 showed that the level of the E2F-pRb complex was significantly reduced or not detectable, whilst the amount of free E2F was found to be higher [11].

Early work showed that the replacement of mutated or deleted *Rb* with the wild-type counterpart resulted in the suppression of tumorigenicity in human osteosarcoma, bladder cancer and prostate carcinoma [13–15]. Further-

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more, injection of the pRb protein into bladder cancer cells in the G1 phase blocked the progression of the cell cycle and hence cell proliferation [16]. Recently, adenoviral-mediated *Rb* gene transfer studies showed that the restoration of pRb suppressed the proliferation of spontaneous mouse pituitary melanotroph tumour, human pancreatic tumour, glioma and breast cancer [17–20]. An anti-proliferative activity was also detected in HeLa cells when they were transfected with the pRb-expressing plasmid [21].

Nevertheless, the effect of the expression of wild-type pRb by *Rb* gene transfer varies in different types of tumours. It has been shown that restoration of pRb function by means of viral vectors expressing pRb or micro-injection of the purified pRb protein inhibited preferentially the growth of the pRb-negative (Rb^-) tumour cells, while most of pRb-positive (Rb^+) tumour cells and normal cells were much less inhibited by exogenous pRb [13,14,22]. However, Fung and colleagues reported that the growth suppression effect of pRb was not tumour-specific [23]. In addition, pRb failed to reverse the malignant phenotype of human breast cancer cell lines [24]. Furthermore, it has been shown that pRb inhibited both p53-dependent and independent apoptosis [25,26].

TP53, another important and extensively studied tumour suppressor gene, has been widely used in cancer gene therapy. It has been shown that the p53 protein expressed by the adenoviral vector (Ad5-*p53*) induced both growth arrest and apoptosis in squamous cell carcinoma of the head and neck [27]. In contrast, we found that the transfection of HeLa and C-33A cells with Ad5-*p53* caused apoptosis, but not G1 arrest, 24 h post transfection [28]. In this study, we constructed an adenoviral vector expressing wild-type pRb (Ad5-*pRb*), sought to explore the potential of using the constructed Ad5-*Rb* in cervical cancer gene therapy, and determined whether an enhanced growth inhibitory effect could derive from the co-transfection of cervical cancer cells with Ad5-*pRb* and Ad5-*p53*.

2. Materials and methods

2.1. Cell lines

All cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD, USA). The human embryonic kidney cell line 293 (passage 31) which was used to propagate and titrate the adenovirus, was cultured in minimum essential medium (MEM) supplemented with 10% (v/v) newborn calf serum (NCS). Cervical cancer cell lines, SiHa (HPV-16 positive), HeLa (HPV-18-positive) and C-33A (containing a *TP53* and *Rb* mutation) were maintained in 1:1 mixture of DME/Ham's F12 plus 5% (v/v) heated-inactivated fetal bovine serum (FBS). The osteosarcoma cell line Saos-2, which has partial deletion of *Rb*, was cultured in McCoys 5A with 15% (v/v) FBS. FBS, NCS and MEM were products of Life Technologies Inc. (Gaithersburg, MD, USA). The culture media McCoys 5A and 1:1 mixture of DME/Ham's F12 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Construction of *Ad5*-Rb

A 3.7 kb *Rb* fragment encoding full-length wild-type pRb was cleaved from the plasmid pCMV-*Rb* (a gift from Dr L. Cao, Department of Microbiology, the University of Hong Kong) by *Bam HI* and *Sca I* digestion, and cloned into the *Bam HI*/*Eco RV* cutting sites of pCA14, an adenovirus type 5 (Ad5) shuttle plasmid, to form pCA14-*Rb*. The restriction enzymes *Bam HI*, *EcoRI*, *Eco RV*, *Hind III* and *Sca I* were products of Life Technologies Inc., and the plasmids pCA14 and pJM17 (a plasmid containing 90% of the Ad5 genome) were from Microbix Biosystems Inc. (Toronto, Ontario, Canada). Plasmid DNAs of pCA14-*Rb* and pJM17 were isolated on a large scale by a standard procedure using alkaline/sodium dodecyl sulphate (SDS). The isolated plasmids were further purified by polyethylene glycol (PEG 6000) precipitation. Recombinant adenovirus (Ad5-*Rb*) expressing pRb were obtained by co-transfection of 293 cells in a monolayer with pJM17 and pCA14-*Rb* by calcium phosphate precipitation according to a method detailed by the supplier. Briefly, a suspension of DNA/calcium phosphate complex was added the monolayer of 293 cells grown in a 60 mm culture dish in the presence of the culture medium. After the addition of the DNA/calcium phosphate complex, the 293 cells were allowed to grow for 10 more hours at 37 °C. The culture medium was then removed, overlaid with 0.5% (w/v) agarose, and incubated at 37 °C for 7–10 days enabling homologous recombination between the pJM17 and pCA14-*Rb* to occur. A total of 48 plaques were found at the end of the incubation period and they were carefully picked using sterile Pasteur pipettes. The plaque containing agarose plug was placed in 0.5 ml of phosphate-buffered saline (PBS) containing 10% (v/v) glycerol and served as a first seed to propagate sufficient quantities of candidate Ad5-*Rb* for initial characterisation. This was done by transfecting 293 cells grown in six-well plates and the harvested 293 cells were used as second seeds.

2.3. Characterisation of pRb-expressing adenovirus

Western blot (WB) analyses and immunohistochemical (IHC) staining were used to characterise Ad5-*Rb*. For WB analysis of Ad5-*Rb*, adenoviral suspension prepared from the second seeds by three freeze and thaw cycles, was used to transfect Saos-2 cells grown in

a six-well plate. The Ad5-*Rb*-transfected cells were harvested 2 days later and lysed in SDS polyacrylamide gel electrophoresis (PAGE) loading buffer (0.05M NaOH, 0.5% (w/v) SDS, 5 mM ethylenediamine tetraacetic acid (EDTA) and 0.025% bromophenol blue). The lysate was denatured at 95 °C for 5 min, separated on a 6% (w/v) gel by SDS-PAGE, electrotransferred to nitrocellulose membrane and reacted with 1/1000 anti-human pRb-specific monoclonal antibody (Ab-5, Oncogene Research Products, Cambridge, MA, USA). This was followed by the detection of the pRb/anti-pRb antibody complex using an anti-mouse secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) kit (Amersham Life Science, Bucks, UK). For IHC staining of pRb protein, Saos-2 cells grown in a 96-well plate were transfected with adenoviruses of the second seeds for 48 h. Avidin–biotin complex (ABC) method using VECTSTAIN ABC kits (Vector Laboratories Inc., Burlingame, CA, USA) in combination with the pRb-specific antibody was performed to detect the pRb expression. After initial characterisation, all candidate Ad5-*Rb* were further purified by three additional rounds of sequential dilution, transfection and screening. The obtained clones were subjected to a final round of characterisation by WB analysis and IHC staining. True pRb-expressing clones were confirmed by the presence of a pRb-specific antibody cross-reacting protein band with a molecular weight of ~110 kDa by WB analysis, and the detection of a brown colour in the nuclei of the Saos-2 cells by IHC staining. The pRb-expressing Ad5-*Rb* were then propagated on a large scale and titrated with a 293 cell monolayer as described by Graham and Prevec in Ref. [29].

2.4. Identification of replication-deficient Ad5-Rb

To ensure that the purified Ad5-*Rb* was replication-deficient, i.e. containing no Ad5-*E1B* gene, polymerase chain reaction (PCR) for the detection of the Ad5-*E1B* DNA was performed according to the method of Zhang and colleagues [30]. The forward and reverse primers for amplifying the Ad5-*E1B* were detailed as follows: Ad5-*E1B*-F (forward) 5′-ATGGAGCGAAGAAACCCATCT-3′, and Ad5-*E1B*-R (reverse) 5′-CTCAATCTGTATCTTCATCGC-3′. DNA (100 ng) obtained from C-33A cells after transfection with Ad5-*Rb* at a multiplicity of infection (MOI) of 50 for 2 days were used as template. Apart from the template, the reaction mixture contained (in a final volume of 50 μl) 5 μl 10×PCR buffer (100 mM Tris/HCl, 15 mM $MgCl_2$ and 500 mM KCl, pH 8.3), 1 μl 10 mM deoxynucleotide triphosphates (dNTPs), 1 μl (20 pmol) Ad5-*E1B*-F, 1 μl (20 pmol) Ad5-*E1B*-R and 0.25 μl (1.25 U) of Taq DNA polymerase (Roche Diagnostic GmbH, Mannheim, Germany). The PCR was carried out using a PTC 100-60 programmable thermal controller (MJ Research Inc., Watertown, MA, USA). Thirty-five cycles of each PCR were performed after an initial denaturation step at 95 °C for 4 min, and each cycle consisted of a denaturation step, annealing step and an extension step at 94, 54 and 72 °C for 1 min each, respectively. This was followed by a final extension step at 72 °C for 10 min and the amplified PCR products were verified by gel electrophoresis using 3% (w/v) NuSieve 3:1 agarose (FMC BioProducts Inc., Rockland, ME, USA). The suitability of the DNA isolated from the Ad5-*Rb* transfected C-33A cells was checked by PCR with the primer set, GH20 and PC04, for amplification of the human *β-globin* gene by yielding a 268 bp PCR product. Conditions for the human *β-globin* gene amplification were essentially the same as for amplifying Ad5-*E1B* except that the annealing temperature was 56 °C. The primer sequences for GH20 (forward) and PC04 (reverse) are: 5′-GAAGAGCCAAGGACAGGTAC-3′ and 5′-CCACTTCATCCACGTTCACC-3′. DNA prepared from the 293 cells was used as the positive control.

2.5. Cell cycle and apoptosis analysis

Cells (5×10^5) grown in a 6-well plate were transfected with different MOIs of Ad5-*Rb* or Ad5-*p53* alone, or in combination. Ad5-*LacZ* was used as the control virus. The transfected cells (including detached cells) were harvested 24 or 48 h later with trypsin/EDTA, washed in PBS, and fixed by the addition of cold 70% (v/v) ethanol. Prior to analysis, the cells were washed and resuspended in PBS. RNase was then added to the cell suspensions at a final concentration of 20 μg/ml and kept at 37 °C for 30 min. Propidium iodide (40 μg/ml) was added and the cells stained for 30 min. The stained cells were analysed with an Epics Elite Esp (Coulter). Five thousand cells were counted and analysed for each sample. The ModFit LT Cell Cycle Analysis Software (Verity Software House, Inc.) was used to analyse the cell cycle events and amount of apoptosis.

2.6. Growth inhibition experiments

The 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrasodium bromide (MTT) assay was used to determine the inhibition of cell growth after the adenoviral transfection [31]. Briefly, 5000 cells were seeded into each well of a 96-well plate and allowed to grow for 24 h. Cells were transfected with different MOIs of Ad5-*Rb*, Ad5-*p53*, Ad5-*LacZ, or* Ad5-*Rb* plus Ad5-*p53*, and cultured for 4 more days. MTT (25 μl) at a concentration of 5 mg/ml was then added to cells in each well and the cells were allowed to grow for 2 more hours. This was followed by the addition of lysis buffer, incubation and measurement of changes in optical density (OD) at 570 nm. Each point of determination represented an average of six

repeated samples of cells grown under the same condition. Adenoviral vector-expressing wild-type human p53 protein (Ad5-*p53*) and the adenovirus control Ad5-*LacZ* were kind gifts from Professor J.A. Roth, MD Anderson Cancer Center, Houston, TX, USA.

2.7. Colony formation in soft agar

A method involving two layers of agarose was used and cells for the colony formation study were harvested by trypsin/EDTA digestion from a 24-well plate (2×10^4 cells/well) after being transfected with different adenoviral constructs for 24 h. The obtained cells were reconstituted to a cell suspension at a density of $0.3–1\times10^6$ cells/ml, and 10 μl of the suspension was placed onto the surface of the bottom layer (0.5% (w/v) agarose in 1×1:1 mixture of DME/Ham's F12) in 60 mm culture dishes. Five millilitres of 0.33% (w/v) low-melting-point agarose in 1×1:1 mixture of DME/Ham's F12 were then added to each dish and gently swirled for even cell distribution. The added agarose was then allowed to set at 4 °C for 10 min and the number of colonies formed in each culture dish was counted after incubation for 3–4 weeks at 37 °C.

2.8. Statistics

Student's paired *t*-test and one way analysis of variance (ANOVA) were used for statistical analyses.

3. Results

3.1. Expression of pRb in *Ad5*-Rb-transfected cervical cancer cells

Prior to the study of pRb expression in the Ad5-*Rb* transfected cells, the relationship between MOI used in the transfection experiments and the percentage of cells being positively transfected with Ad5-*Rb* was carried out. The results obtained from the IHC staining showed that the number of Rb-positive cells approached 100% in C-33A, SiHa and HeLa cells upon transfection with Ad5-*Rb* for 24 h (data not shown). It is for this reason that western blot analyses of pRb in cell-free extracts prepared from C-33A, SiHa and HeLa cells after Ad5-*Rb* transfection for 24 h at the said MOIs were performed. Our WB analyses revealed strong expression of pRb in these three tested cervical cancer cell lines (Fig. 1). In contrast, neither the untreated medium control nor cervical cancer cells transfected with the adenoviral control, Ad5-*LacZ* induced pRb expression (Fig. 1). However, levels of pRb expressed by the cervical cancer cells that were co-transfected with Ad5-*Rb* and Ad5-*p53* were found to be variable as compared to those transfected with Ad5-*Rb* alone (Fig. 1).

3.2. *Ad5*-Rb altered cell cycle events in Saos-2 and cervical cancer cell lines

When transfected with Ad5-*Rb* at MOIs of 50 and 200 for 48 h which achieved 80 and 100% Ad5-*Rb* transfection, flow cytometric analyses showed that Ad5-*Rb* not only induced cell cycle changes in the G1 phase of Saos-2 cells, but also an increase in the size of the G1 peak. At MOIs of 50 and 200, 69.2 and 80.3% of the Ad5-*Rb*-transfected Saos-2 cells were detected in G1 phase, i.e. a G1 block (data not shown). An increase in the size of the G1 peak was also found in C-33A and SiHa cells after Ad5-*Rb* transfection at MOIs of 10 and 20, respectively. However, a change in the distribution of HeLa cells at different cell cycle phases was not observed when they were transfected with Ad5-*Rb* at a MOI of 200 (Table 1).

3.3. Reduction of colony formation in Saos-2 cells after *Ad5*-Rb transfection

The number of colonies formed by the Saos-2 cells after the transfection of Ad5-*Rb* at a MOI of 20 was

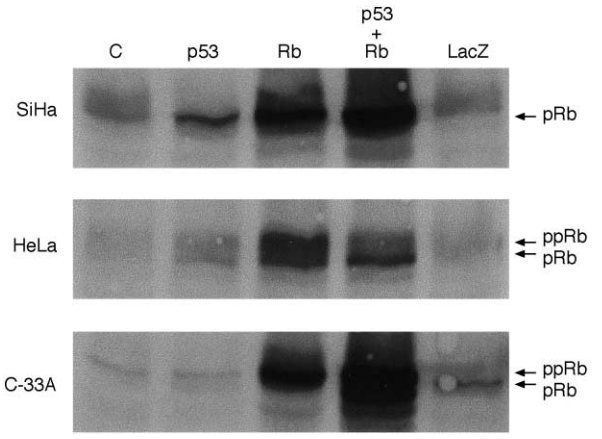

Fig. 1. Determination of pRb expression by western blot analysis in cervical cancer cells SiHa (upper panel), HeLa (centre panel) and C-33A (bottom panel) with the medium control (C), after the transfection with Ad5-*p53* (p53), Ad5-*Rb* (Rb), Ad5-*Rb* and simultaneous co-transfection with Ad5-*p53* (p53 + Rb), and the control Ad5-*LacZ* (Lac Z) for 24 h. pRb and ppRb denote the hypophosphorylated and hyperphosphorylated form of the retinoblastoma protein, respectively.

Table 1
Distribution (percentage) of cervical cancer cells in the G1 phase after transfection with the adenovirus control Ad5-*LacZ* and the Ad5-*Rb* for 24 h

	SiHa (MOI = 20)	HeLa (MOI = 200)	C-33A (MOI = 10)
Medium control	53.0	50.9	52.9
Ad5-*LacZ*	47.3	47.4	53.8
Ad5-*Rb*	72.4	47.6	64.3

MOI, multiplicity of infection.

found to be significantly lower than those transfected with the adenovirus control (Ad5-*LacZ*) and the medium control ($P<0.05$). This finding suggested the inhibition of colony formation in Ad5-*Rb*-transfected Saos-2 cells. For every 1000 cells seeded in the colony formation experiments, the number of colonies formed by Saos-2 cells being transfected with Ad5-*Rb,* Ad5-*LacZ* and the medium control, were found to be 158.8 ± 45.5, 207.7 ± 34.0, and 219.7 ± 40.2, respectively (Table 2). However, no inhibition of colony formation was detected in the SiHa, HeLa and C-33A cells after the transfection of Ad5-*Rb* when the number of colonies formed in the Ad5-*Rb*-transfected cells were compared with those transfected with Ad5-*LacZ* and the untreated medium control (Table 2).

3.4. Lack of inhibition of growth in *Ad5*-Rb-*transfected cervical cancer cells*

Growth studies of SiHa, HeLa and C-33A cervical cell lines after transfection with Ad5-*Rb* alone or in combination with Ad5-*p53* for 4 days were determined by the MTT method. Compared with the medium control, only a weak inhibition of growth was noted. Moreover, the inhibition of growth in both the SiHa and HeLa cells after the Ad5-*Rb* transfection was shown to be significantly less than those transfected with Ad5-*LacZ* ($P<0.05$, Fig 2a and b). On the other hand, the growth inhibitory effect was essentially the same for C-33A cells after transfection with Ad5-*LacZ* or Ad5-*Rb* (Fig. 2c). In contrast, a stronger growth inhibition was detected in all three cervical cancer cell lines after transfection of Ad5-*p53* at different MOIs, i.e. Ad5-*p53* versus Ad5-*LacZ*, ($P<0.05$).

When co-transfected with Ad5-*p53*, the inhibitory effect of Ad5-*Rb* on the growth of both SiHa and HeLa cells was found to be weaker than that of Ad5-*LacZ* co-transfected with Ad5-*p53* (Ad5-*Rb*+Ad5-*p53* versus Ad5-*LacZ*+Ad5-*p53*, $P<0.05$). In the case of C-33A cells, the difference in the inhibition of growth between these two co-transfection groups was not statistically significant ($P>0.05$). Fig. 2a–c depicted clearly that no enhancement of growth inhibition was observed from SiHa and C-33A cells after co-transfection with Ad5-*p53* and Ad5-*Rb* compared with those transfected with Ad5-*p53* alone (Ad5-*p53*+Ad5-*Rb* *vs.* Ad5-*p53*, $P>0.05$). In contrast, a stronger growth inhibitory effect was observed in the HeLa cells upon the Ad5-*Rb* and Ad5-*p53* co-transfection when compared with that

Table 2
Number of colonies formed by cervical cancer cells and Saos-2 cells after adenovirus transfection

	Medium control	Ad5-*LacZ*(a)	Ad5-*Rb* (b)
SiHa	76.3±12.2	37.3±10.0	65.3±19.1
HeLa	275.7±33.2	228.7±47.6	279.0±29.6
C-33A	108.7±14.2	38.0±7.9	42.7±8.2
Saos-2	291.7±40.1	207.7±34.1	158.8±45.5

Each value is expressed as mean±standard deviation (S.D.) of three independent experiments. Student paired *t*-test was used for data analysis. The *P* values for a versus b, i.e. transfection of Ad5-*LacZ* versus Ad5-*Rb*, were >0.05, >0.05, >0.05 and <0.05 for SiHa, HeLa, C-33A and Saos-2 cells, respectively.

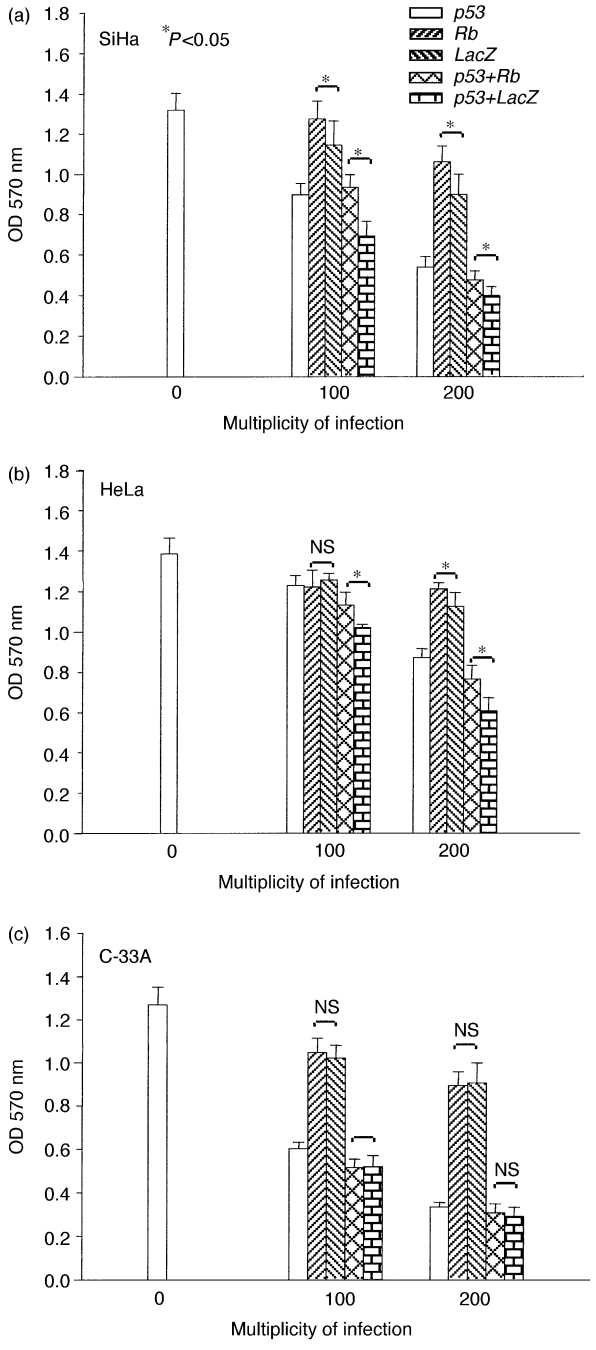

Fig. 2. The growth inhibition of SiHa (a), HeLa (b) and C-33A (c) cells as determined by the MTT assay after transfection with Ad5-*Rb*, Ad5-p53 and Ad5-*LacZ* alone, and in combinations at multiplicities of infection (MOIs) as indicated for 4 days.

Table 3
Distribution (percentage) of cervical cancer cells in sub-G1 peak, i.e. undergoing apoptosis, after transfection with Ad5-*p53*, Ad5-*Rb*, and Ad5-*LacZ* alone, and Ad5-*p53* in combination with Ad5-Rb or Ad5-*LacZ*

	SiHa (MOI = 20)	HeLa (MOI = 200)	C-33A (MOI = 10)
Medium control	0.62±0.80	3.91±0.33	0.72±0.06
Ad5-*LacZ*	1.09±0.10	6.45±1.04	0.70±0.03
Ad5-*Rb*	0.83±0.21	3.42±0.33	0.79±0.18
Ad5-*p53*	27.20±6.99	32.54±4.00	50.91±6.80
Ad5-*p53*±Ad5-*Rb* (a)	12.16±4.13	35.76±2.75	33.21±1.56
Ad5-*p53*±Ad5-*LacZ* (b)	26.23±4.20	42.84±6.51	43.41±1.90

MOI, multiplicity of infection. Each value is expressed as mean ±standard deviation (S.D.) of three independent experiments. Student paired *t*-test was used for data analysis. The *P* values for a versus b, i.e. transfection of Ad5-*p53* + Ad5-*Rb* versus Ad5-*p53* + Ad5-*LacZ*, were 0.0023, 0.23 and 0.0155 for SiHa, HeLa and C-33A cells, respectively.

of Ad5-*p53* alone (Ad5-*p53* + Ad5-*Rb* versus Ad5-*p53*, $P < 0.05$). However, Ad5-*p53* + Ad5-*Rb* exhibited less growth inhibition than that of Ad5-*p53* + Ad5-*lacZ* ($P < 0.05$).

3.5. Inhibition of the p53-dependent apoptosis in cervical cancer cells by *Ad5*-Rb

Flow cytometric analyses were performed with HeLa, C-33A and SiHa cells after transfection with Ad5-*Rb*, Ad5-*p53* alone, or in combination (Ad5-*Rb* + Ad5-*p53*) for 24 and 48 h, respectively. The rationale of determining apoptotic events at the said time was based on results obtained from our pilot study (data not shown) and earlier findings [28] that HeLa and C-33A, and SiHa cells induced apoptosis after transfection of these cells with Ad5-*p53* alone or in combination with Ad5-*Rb*, for 24 and 48 h, respectively. Data collected from the present study showed that apoptosis was readily detected in all three tested cervical cancer cell lines after the Ad5-*p53* transfection. This was evidenced by the presence of sub-G1 peaks in the flow cytometric measurements (Table 3 and Figs. 3–5). However, the sub-G1 peak was not detected in the Ad5-*Rb*-transfected cells. On the contrary, the size of the sub-G1 peak was found to be smaller in the SiHa and C-33A cells after transfection with Ad5-*p53* and Ad5-*Rb* compared with those transfected with Ad5-*p53* plus Ad5-*LacZ*, and Ad5-*p53* alone, respectively. On the other hand, a larger sub-G1 peak was detected in the HeLa cells after the Ad5-*p53* and Ad5-*Rb* co-transfection, but the sub-G1 peak was smaller than that in HeLa cells transfected with Ad5-*p53* and Ad5-*LacZ* ($P > 0.01$). We also noted the occurrence of a relatively large G2/M peak in the SiHa cells after the Ad5-*LacZ* transfection for 48 h.

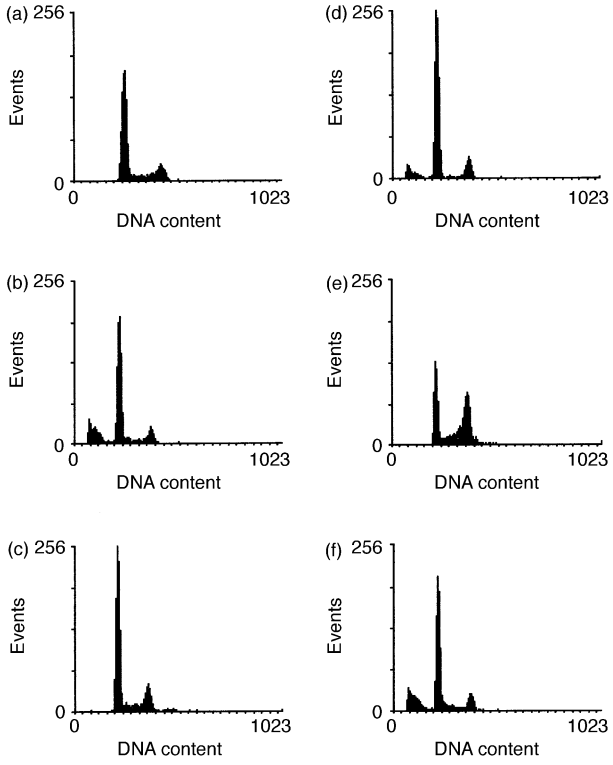

Fig. 3. Analysis of apoptotic events in SiHa cells by flow cytometry 48 h after transfection with Ad5-*p53*, Ad5-*Rb* and Ad5-*LacZ* alone at a multiplicity of infection (MOI) of 10, and Ad5-*p53* in combination with Ad5-*Rb* or Ad5-*LacZ* at a MOI of 20 each. In addition to the medium control (a), transfection of SiHa cells with Ad5-*p53* (b), Ad5-*Rb* (c), Ad5-*p53* + Ad5-*Rb* (d), Ad5-*LacZ* (e), and Ad5-*p53* + Ad5-*LacZ* (f) are also presented.

4. Discussion

In this study, we explored the possibility of restoring the function of *Rb* by the transfection of Ad5-*Rb* into three cervical cancer cell lines. To achieve this goal, we constructed the pRb-expressing Ad5-*Rb* under the control of the cytomegalovirus (CMV) immediate early gene promoter. The normal functioning of our constructed pRb-expressing adenovirus (Ad5-*Rb*) was verified by the fact that transfection of Ad5-*Rb* into Saos-2 cells for 48 h resulted in both G1 arrest and inhibition of colony formation. Furthermore, an increase of the cell population in the G1 phase was noted when SiHa and C-33A cells were transfected with Ad5-*Rb* for 24 h. Nevertheless, the cell cycle of HeLa cells was found not to be affected by the Ad5-*Rb* transfection. In addition, no inhibition of growth *in vitro* was detected when SiHa, HeLa and C-33A cells were transfected with Ad5-*Rb*. In fact, transfection with Ad5-*Rb* alone, and in combination with Ad5-*p53* showed a similar or weaker growth inhibitory effect in these three cervical cancer cell lines than those transfected with the control virus Ad5-*LacZ* alone, and Ad5-*LacZ* plus Ad5-*p53* (Fig. 2a–c). As

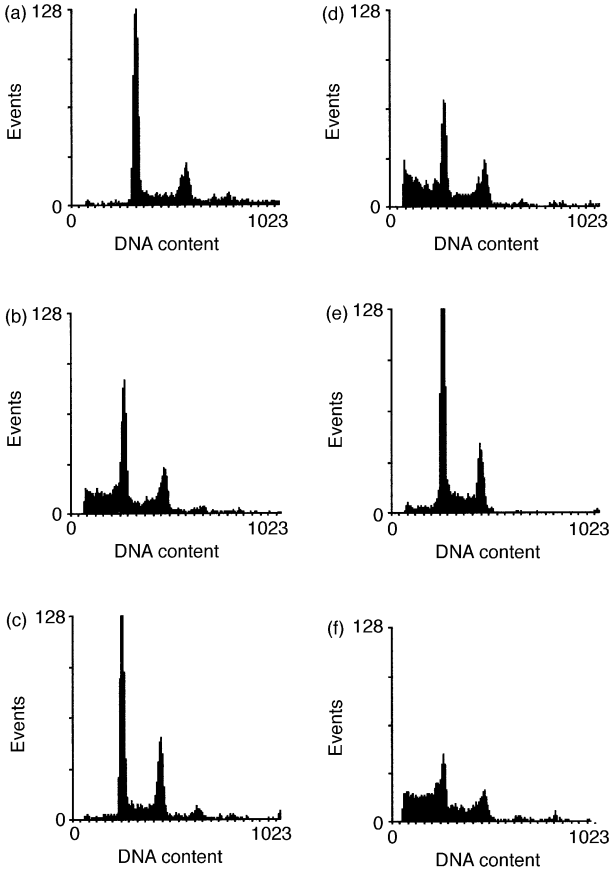

Fig. 4. Analysis of apoptotic events in HeLa cells by flow cytometry 24 h after transfection with Ad5-*p53*, Ad5-*Rb* and Ad5-*LacZ* (the control virus) alone at a multiplicity of infection (MOI) of 200, and Ad5-*p53* in combination with Ad5-*Rb* or Ad5-*LacZ* at a MOI of 200 each. Apart from the medium control (a), transfections of HeLa cells with Ad5-*p53* (b), Ad5-*Rb* (c), Ad5-*p53*+Ad5-*Rb* (d), Ad5-*LacZ* (e), and Ad5-*p53*+Ad5-*LacZ* (f) are also shown.

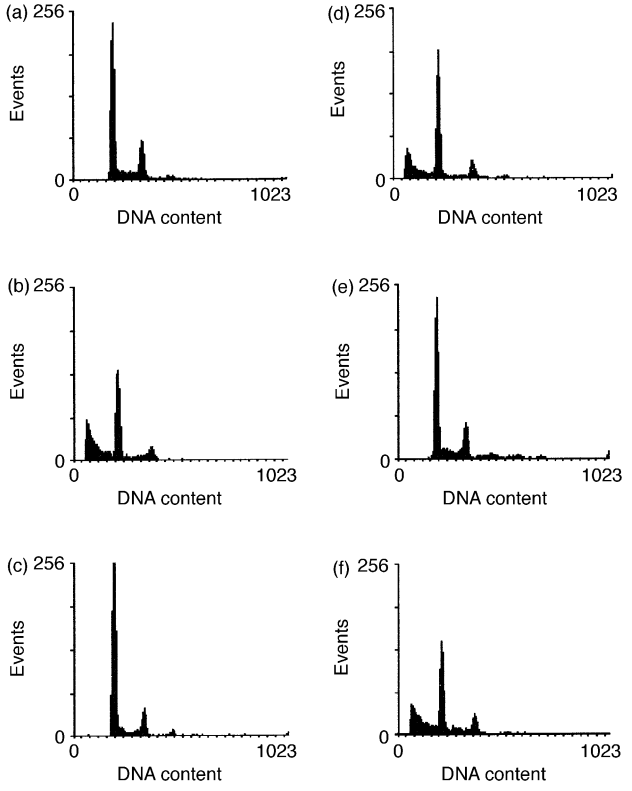

Fig. 5. Analysis of apoptotic events in C-33A cells by flow cytometry 24 h after transfection with Ad5-*p53*, Ad5-*Rb* and Ad5-*LacZ* alone at a multiplicity of infection (MOI) of 20, and Ad5-*p53* in combination with Ad5-*Rb* or Ad5-*LacZ* at a MOI of 20 each. In addition to the medium control (a), transfections of C-33A cells with Ad5-*p53* (b), Ad5-*Rb* (c), Ad5-*p53*+Ad5-*Rb* (d), Ad5-*LacZ* (e), and Ad5-*p53*+Ad5-*LacZ* (F) are also depicted.

shown by the number of colonies formed (Table 2), it also appeared that Ad5-*Rb* did not suppress the colony formation in these three cervical cell lines. The exact cause for the lack of inhibitory effect on cell growth and colony formation in the Ad5-*Rb*-transfected cervical cancer cells remains to be determined.

The growth inhibitory effect of pRb has previously been shown to be variable and incomplete [5,13,23,24]. Although an early study demonstrated growth inhibition in Saos-2 cells after the restoration of pRb [13], it was subsequently reported that the pRb restoration did not lead to growth arrest in Saos-2 cells [23,32]. It has also been reported that overexpression of *Rb* cDNA suppressed the proliferation of HeLa cells [21]. However, no inhibition of growth was observed in the present study when HeLa cells were transfected with Ad5-*Rb*. As the level of pRb expressed in all Ad5-*Rb*-transfected cervical cancer cell lines remained relatively high (Fig. 1), it appears that other undefined factors may also be involved in determining the normal pRb function *in vivo*.

However, flow cytometric analyses showed that the apoptosis caused by the p53 expressing adenovirus Ad5-*p53* was partially inhibited by Ad5-*Rb*. A smaller sub-G1 peak was observed when the SiHa and C-33A cells were co-transfected with Ad5-*p53* and Ad5-*Rb* compared with those transfected with Ad5-*p53* alone (Table 3, and Figs. 3 and 5). Although a larger sub-G1 peak was noted in the HeLa cells transfected with Ad5-*p53* and Ad5-*Rb* compared with those transfected with Ad5-*p53*, the sub-G1 peak was smaller than that of those transfected with Ad5-*p53* plus Ad5-*LacZ* (Table 3 and Fig. 4). The apoptotic data are consistent with the growth inhibition experiments (Fig. 2). Based on these findings, it appears that transfection of adenovirus expressing full-length *Rb* may not be a good strategy for use in cervical cancer gene therapy, and the simultaneous transfection of Ad5-*pRb* and Ad5-*p53* adds no advantage to increase the effectiveness of Ad5-*p53*-mediated gene therapy.

Our observation of the attenuation of p53-induced apoptosis by pRb was in line with previous reports that pRb inhibited apoptosis [25,26], and induction of both p53-dependent and p53-independent cell death was detected as a result of loss of pRb [33]. It has recently

been shown that the cleavage of 42 amino acids from the C-terminus of pRb by ICE (interleukin 1β converting enzyme)-like protease is an important event in the apoptosis [34]. Evidence obtained from these studies suggested that pRb could be an anti-apoptotic factor. In addition, E2F, the transcription factor which binds to the hypophosphorylated pRb, has been implicated as the mediator of pRb function in cell cycle regulation and E2F induces cell apoptosis independent of p53 [35,36]. It is possible that the inhibition of apoptosis by pRb is mediated through its binding with E2F and the active form of pRb may therefore not be useful in gene therapy owing to its ability to induce tumour cells that are resistant to apoptosis.

Although previous studies showed that adenovirus-mediated *Rb* gene transfer inhibited tumour formation and the proliferation of vascular smooth muscle cell [17–20], the growth and tumorigenicity inhibitory effect of pRb varied considerably [5,23,37]. A truncated form of pRb, i.e. pRb^{94}, with 112 amino acids removed from its N-terminus, has recently been constructed and used in a study of tumour growth inhibition. A stronger growth inhibitory effect was obtained with the truncated pRb^{94} when compared with the full-length of pRb^{110} [37,38]. In addition, pRb^{94} was found to have a longer half-life, accumulate rapidly to high level and remain active [37]. Most important of all, pRb^{94} induced apoptosis [38]. Hence, the cDNA coding the truncated pRb^{94} could be a better choice than the full-length *Rb* for use in gene therapy and the potential usefulness of the truncated pRb^{94} in cancer gene therapy merits further investigation.

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