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European Journal of Cancer 36 (2000) 249–256

European Journal of Cancer

www.elsevier.com/locate/ejconline

Changes in p21^{WAF1}, pRb, Mdm-2, Bax and Bcl-2 expression in cervical cancer cell lines transfected with a p53 expressing adenovirus

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Received 22 June 1999; received in revised form 13 September 1999; accepted 20 September 1999

Abstract

The aim of this study was to provide some insights into the molecular mechanisms involved in p53-dependent apoptosis and growth arrest. Changes in the levels of p53 protein and proteins regulated by p53 were studied in relation to events of the cell cycle and apoptosis in cervical cancer cell lines upon transfection with a p53 expressing adenovirus (Ad5-*p53*). The post-transfection level of p53 protein in SiHa cells was found to be unchanged during the 24–48 h period. In contrast, the level of p21^{WAF1} protein was shown to increase to its highest level at 24 h, and decreased gradually up to 48 h after the Ad5-*p53* transfection. We further noted that the increase of p21^{WAF1} was accompanied by G1 arrest at 24 h and the decrease of p21^{WAF1} was associated with apoptosis at 36–48 h after transfection. An anti-p21^{WAF1} antibody cross-reactive protein band of approximately 14 kDa was observed in HeLa and C-33A cells when these cells were committed to apoptosis upon Ad5-*p53* transfection. In SiHa cells, phosphorylation of pRb was inhibited during the early stage of Ad5-*p53* transfection. This was followed by the cleavage of pRb. However, Ad5-*p53* transfection did not change the levels of Bax and Bcl-2 proteins. Our results suggested that, Bax and Bcl-2 may not be important for the apoptosis of these cells, whereas cleavage of Rb, and the decrease of p21^{WAF1} could play important roles in p53-dependent apoptosis.

Keywords: p53; p21^{WAF1}; pRb; Mdm2; Bax/Bcl-2; Growth arrest/apoptosis; Adenoviral vector; Cervical cancer cell lines

1. Introduction

TP53 is an important tumour suppressor gene. However, in more than 85% of cervical cancer cases, p53 is inactivated by the E6 protein of human papillomavirus (HPV) [1]. Loss of p53 function leads to impairment in the control of the cell cycle and tumour formation. Restoration of wild-type (wt) p53 in p53 mutated and functionally inactivated p53 tumours slows tumour cell growth *in vitro* and the growth of tumour xenografts in nude mice [2–4]. Recently, several studies also showed that ectopic p53 expression causes tumour cells to undergo apoptosis [5,6]. In normal cells, the expression of p53 is induced by genotoxic stimulation such as irradiation and chemotherapeutic agents. Upon receiving external stimulation, especially the stimulation that imposes damage on cellular DNA, an increase in the level of wt-p53 is seen and this results in cell cycle arrest or apoptosis [2]. By direct and specific binding to DNA, p53 regulates the expression of several molecules including p21^{WAF1}, Bax and Mdm-2 [7]. The effect of p53 on cell cycle regulation acts partly through its downstream molecule p21^{WAF1}. The latter inhibits the cyclin-dependent kinases (cdks) that determine the phosphorylation of pRb [8]. Hypophosphorylated pRb, which exists mainly in the G1 phase of the cell cycle, binds to and inhibits the activity of transcription factors belonging to the E2F family [9], and this is largely responsible for the subsequent growth arrest [7].

However, the exact molecular events leading to p53-induced apoptosis are still unclear. p53 upregulates molecules such as Bax which regulates cells to undergo apoptosis [10]. Bax is able to form a heterodimer with

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0959-8049/99/$ - see front matter
PII: S0959-8049(99)00247-6

Bcl-2, an anti-apoptotic factor, and plays a critical role in the regulation of apoptosis [10,11]. In contrast, Bax is not an absolute prerequisite for p53-dependent apoptosis [12,13]. Recently, it has been reported that both $p21^{WAF1}$ and pRb inhibit apoptosis [3,14,15]. p53 transactivates Mdm-2 which forms a complex with and inhibits the transcriptional activity of p53 [16]. p53 induction stimulates both the pro-apoptotic factors such as Bax and anti-apoptotic factors such as $p21^{WAF1}$, pRb as well as Mdm-2 and it is not fully understood how a cell decides to undergo apoptosis or growth arrest.

$p21^{WAF1}$ seems to play an important role in determining whether a cell should undergo apoptosis or growth [17–19]. Disruption of $p21^{WAF1}$ function can result in cells more prone to apoptosis [18]. It has also been reported that transfection of Ad5-*p53* induces apoptosis in melanoma cells which have little $p21^{WAF1}$ whilst it induces a moderate inhibition of growth of primary vascular smooth muscle cells which have high $p21^{WAF1}$ levels [19]. We, therefore, examined the levels of some of these molecules following transfection of Ad5-*p53* into three cervical carcinoma cell lines in order to elucidate further how the cell decides on p53-dependent apoptosis versus cell cycle arrest.

2. Materials and methods

2.1. Cell lines and antibodies

The cell line 293 was used in this study to propagate and titrate adenovirus. Human cervical cancer cell lines including SiHa (HPV16 positive), HeLa (HPV18 positive) and C-33A (containing a p53 codon 273 CGT to TGT mutation) were obtained from The American Type Culture Collection (Rockville, MD, USA). Cervical cancer cell lines were maintained in a 1:1 mixture of DME/Ham's F12 (Sigma Chemical Co., St Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Propagation and titration of the replication-deficient adenoviruses were performed according to the method described by Graham and Prevec [20]. The replication-deficient adenoviruses Ad5-*p53*, in which p53 expression was under the control of the cytomegalovirus promoter, and Ad5-*LacZ*, in which *Escherichia coli* β-galactosidase is expressed, were kind gifts of J. Roth from Introgen Co. Ltd (Houston, TX, USA).

Antibodies used in this study included $p21^{WAF1}$ (Pharmingen, San Diego, CA, USA), p53 (pAb1801) and Rb (Ab-5) (Oncogene Research Products, Cambridge, MA, USA), Bax (B9), Bcl-2 and Mdm-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and human β-actin (Sigma Chemical Co.). All these antibodies were mouse anti-human monoclonal antibodies (MAb).

2.2. Western blotting of p53, $p21^{WAF1}$, Bax, Bcl-2, Mdm-2 and pRb

Human cervical cancer cell lines including SiHa, HeLa and C-33A were studied. The cells grown in 6-well culture dishes were transfected with Ad5-*p53* or control virus Ad5-*LacZ*. The transfected cells (both detached and undetached cells) were harvested 24 h after transfection and lysed in buffer containing 50 mM Tris–Cl pH 8.0, 150 mM NaCl, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml of aprotinin and 1% NP40. The supernatant of the cellular lysate was collected after centrifugation at $10\,000\times g$. The protein contents of the lysate were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as internal control and reference to quantify the amount of protein loaded. Forty micrograms of denatured protein was then separated on 6% (for pRb), 10% (for p53, Mdm-2 and β-actin) or 15% (for $p21^{WAF1}$, Bax and Bcl-2) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to nitrocellulose membranes by electrophoresis. The nitrocellulose membranes were incubated with the primary antibody at 1 μg/ml for 16 h at room temperature (Amersham Life Science, Amersham, Bucks, UK), and then the second antibody conjugated to horseradish peroxidase for 1000-fold dilution. The specific antibody reactive bands were detected using an ECL kit (Amersham Life Science).

2.3. Cell cycle analysis

Cervical cancer cells grown in 6-well plates (5×10^5) were transfected with adenovirus at different multiplicity of infections (MOIs). Cells (including the detached cells) were harvested 24 h later with trypsin/EDTA, washed in cold phosphate buffered saline (PBS) and fixed by the addition of cold 70% ethanol. Prior to analysis, the cells were washed and resuspended in PBS. RNase was then added to 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 stained for 10 min. The stained cells were analysed with FACScan (Becton Dickinson, USA). Ten thousand cells were counted and analysed for each sample. SOBR (sum of broadened rectangles) software was used to analyse cell cycle and Lysis II for apoptosis.

3. Results

3.1. Ad5-p53 was highly infectious and p53 protein was actively expressed

Upon transfection with Ad5-*p53*, the expression of p53 was readily detected in all three cervical cancer cell

lines studied (Fig. 1a, b and Fig. 2a). In HeLa and C-33A cells which contain wt and codon 273 mutant p53, respectively, the increase in the level of p53 was detected when increasing MOI of Ad5-*p53* was used to transfect. Owing to the presence of the mutated p53 in C-33A cells, the immunoreactive p53 band was detected by PAb1801 in both the untreated control and the virus control lanes. However, the exogenous p53 level was found to be much higher than the endogenous mutated p53, and the endogenous p53 was shown to migrate faster than that of exogenous p53 (Fig. 1b). Apart from the 53 kDa band, several bands with molecular weights less than 53 kDa were also observed by the Western blot analysis of the p53 protein which could be degradation products.

3.2. p53 expressed by the adenovirus upregulated $p21^{WAF1}$ and Mdm-2 proteins, decreased the phosphorylation of pRb, and did not affect Bax and Bcl-2 levels

To determine the roles of molecules responsible for p53 function, changes in the levels of proteins such as $p21^{WAF1}$, Mdm-2 and Bax regulated by p53 were studied by Western blot analyses after cervical cancer cells were transfected with Ad5-*p53*. In SiHa cells, the $p21^{WAF1}$ protein level was found to increase and the increase corresponded to the increase of p53 protein upon Ad5-*p53* transfection for 24 h, whereas the Ad5-*LacZ* transfection did not induce p53 and $p21^{WAF1}$ (Fig. 2a). However, the $p21^{WAF1}$ protein was shown to decrease with an increase in the p53 protein in both HeLa and C-33A cells (Fig. 1a, b). In addition, a smaller molecular weight protein band of ~14 kDa, which reacted with the $p21^{WAF1}$ MAb, was detected in both of these two cervical cancer cell lines. This smaller band might be cleaved $p21^{WAF1}$.

Western blot analyses of a panel of proteins associated with growth arrest and/or apoptosis have been performed with SiHa cells transfected by Ad5-*p53* at a MOI of 20 for 24, 36 and 48 h. Human β-actin Western blot showed that the amount of protein applied to each

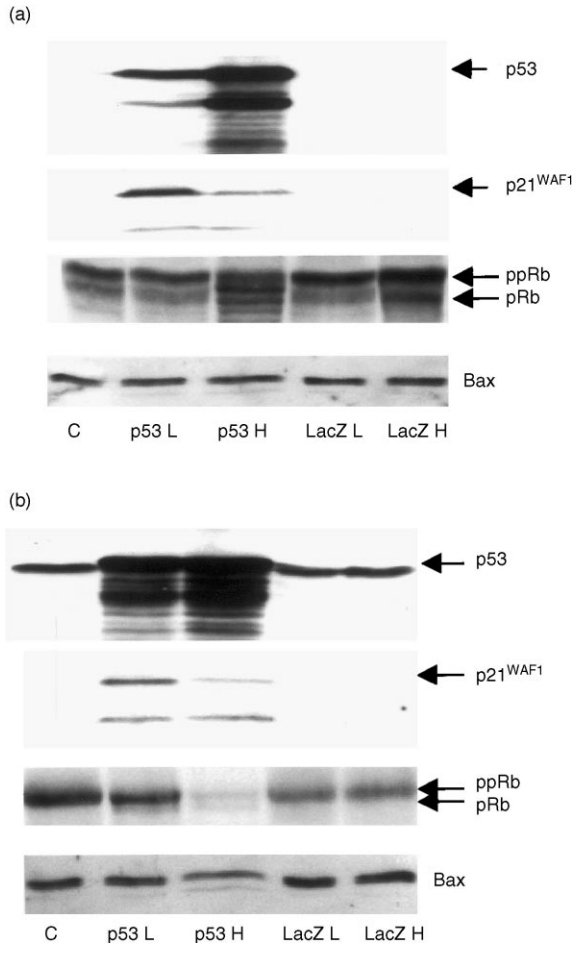

Fig. 1. Western blot analysis of p53, $p21^{WAF1}$ pRb and Bax 24 h after transfection in cervical cancer cells HeLa (a) at 50, 200 MOI; and C-33A (b) at 5, 50 MOI with Ad5-*p53* (p53) and Ad5-*LacZ* (LacZ), the virus control. L or H means transfection at low or high MOI.

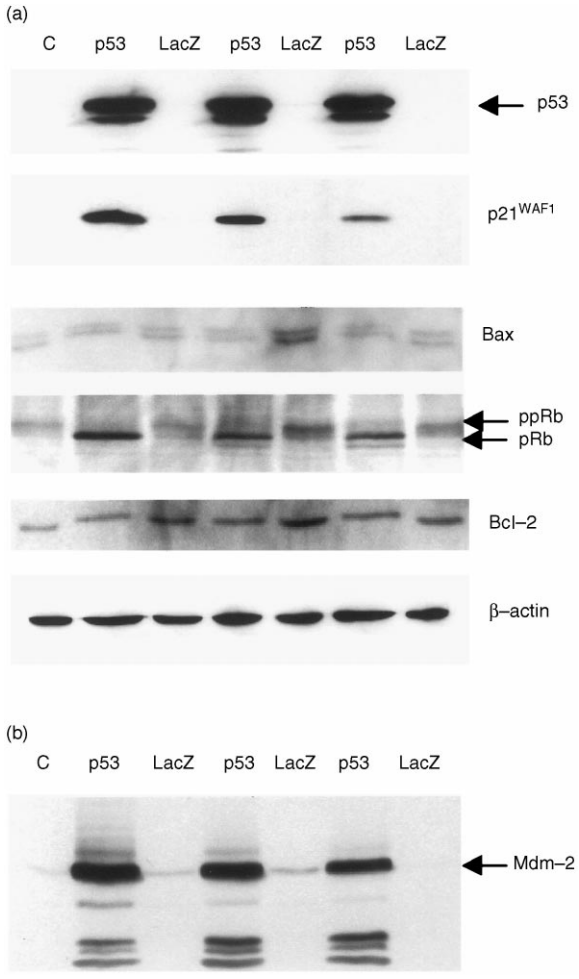

Fig. 2. Western blots of p53, $p21^{WAF1}$, pRb, Bax, Bcl-2 and β-actin (a), as well as Mdm-2 (b) at 24 (lanes 2, 3), 36 (lanes 4, 5) and 48 h (lanes 6, 7) after transfection with Ad5-*p53* (p53) at a MOI of 20 in SiHa cells. The untreated control (lane 1) and the virus control (LacZ) samples were arranged side by side.

lane was essentially the same. Moreover, only one protein band was detected in each lane suggesting that there was no degradation of β-actin (Fig. 2a). p53 protein was highly expressed and did not fluctuate with time after transfection. However, $p21^{WAF1}$ and Mdm-2 were found to be strongly upregulated by p53, and $p21^{WAF1}$ reached its highest level at 24 h, and started to decrease at 36 h, reaching its lowest level at 48 h. During this period, a 4.4-fold decrease in $p21^{WAF1}$ was detected. A 90 kDa Mdm-2 band was observed upon exogenous p53 expression, and this band was slightly weaker at 36 h compared with that at 24 h, and this decrease of Mdm-2 protein continued at 48 h. Besides the 90 kDa band, smaller molecular weight bands were also detected, that were not found in the virus control group (Fig. 2b). Western blot analysis of pRb revealed the occurrence of an intensive lower molecular weight band indicative of hypophosphorylation of pRb, and the higher molecular weight band representing the hyperphosphorylated form of pRb became weaker upon Ad5-*p53* transfection. When the transfection was performed at MOI of 20, almost no hyperphosphorylated pRb was observed in SiHa cells 24 h post-transfection (Fig. 2a). In addition, protein bands with apparent molecular weights of less than 110 kDa were detected by the anti-human pRb antibody 36 h after Ad5-*p53* transfection, and these smaller bands became stronger at 48 h post-transfection. The hypophosphorylated pRb was also detected in HeLa and C-33A cells after a high MOI of Ad5-*p53* transfection (Fig. 1a, b). The smaller bands of pRb were again detected in HeLa cells, but not in C-33A. In C-33A, the pRb band was weak even when transfected with a high MOI. Although both Bax (in all three cell lines) and Bcl-2 (in SiHa cells) were detected, the levels of these two proteins were found to remain unchanged upon the Ad5-*p53* transfection (Fig. 1a, b and Fig. 2a).

3.3. Exogenous p53 caused both growth arrest and apoptosis in cervical cancer cell lines

To establish a relationship between the changes in the various molecules and the cell cycle or apoptotic pathways, the proportion of cells entering different phases of the cell cycle and the amount of apoptosis in the transfected cell population were studied by flow cytometric analysis. Our results showed that after transfection with Ad5-*p53* at a MOI of 20 for 24 h, the percentage of SiHa cells in the G1 phase was increased from 53.0% to 91.9% and the hypodiploid (sub-G1) peak representing apoptotic cells was undetectable. The proportion of cells at the sub-G1 peak was found to increase to 16.0% and 65.6%, 36 h and 48 h, respectively, after Ad5-*p53* transfection (Fig. 3). When HeLa and C-33A cell lines were transfected with Ad5-*p53* at MOI of 200 and 50, respectively, sub-G1 peaks were detected 24 h after the transfection (Fig. 4). The sub-G1 peaks were found to account for 61.0% and 70.4% of the total cell population of HeLa and C-33A, respectively. To confirm the result of apoptosis observed in the flow cytometric analysis, DNA fragmentation analysis was performed. Typical DNA ladders were detected in SiHa cells 36 and 48 h after transfection of Ad5-*p53*, whilst the viral control group did not show any DNA laddering (Fig. 5). In addition, the inhibition of growth of these three cervical cancer cell lines, namely SiHa, HeLa and C-33A, was also observed after Ad5-*p53* transfection (data not shown).

4. Discussion

In this study, we showed that the protein expressed by Ad5-*p53* was much higher than the endogenous mutated p53 in the C-33A cell line at a MOI of 5. This is probably due to the fact that the expression of p53 in the Ad5-*p53* construct is under the control of a strong cytomegalovirus promoter. The p53 levels in the cells transfected with Ad5-*p53* were, in fact, supraphysiologically high. Under this condition, several smaller protein bands showed p53 antibody cross-reactivity. It has been shown that p53 can be cleaved to produce p35 in the presence of double-stranded DNA, and that p35 could further cleave p53 at the N- and C-terminals to produce p50 and p40 [21]. Thus, the smaller bands found in this study could be cleaved fragments of p53.

In the three cell lines studied here, p53 is inactivated either by functional inactivation via the E6 protein of human papillomavirus (SiHa and HeLa) [1], or by mutation (C-33A). The restoration of p53 function with a p53-expressing adenovirus resulted in G1 arrest and/or apoptosis in these cells. Ad5-*p53* transfection at a MOI of 20 for 24 h caused a proportion of SiHa cells to arrest in G1. 12 h later, i.e. 36 h after transfection, 16.0% of SiHa cells were committed to undergo apoptosis, and the percentage of apoptotic cells increased rapidly to 65.6% at 48 h after transfection. In contrast, the transfection of HeLa and C-33A cells with Ad5-*p53* did not cause G1 arrest, but apoptosis 24 h post-transfection.

$p21^{WAF1}$ seems to play an important role in apoptosis. It has been shown that when a colorectal cell line (D line), which was prone to commit apoptosis, was fused with another colorectal cell line (A line), which was prone to growth arrest, the resulting fused cell line was prone to commit apoptosis upon p53 induction [18]. It was, hence, proposed that genetics of the cell determined the choice of growth arrest and apoptosis. Moreover, Polyak and colleagues [18] found that the A line behaved as the D line if the $p21^{WAF1}$ function of the A line was disrupted, suggesting that $p21^{WAF1}$ protected cells from apoptosis. Gorospe and colleagues [22] found that Ad5-*p53* induced apoptosis in human melanoma

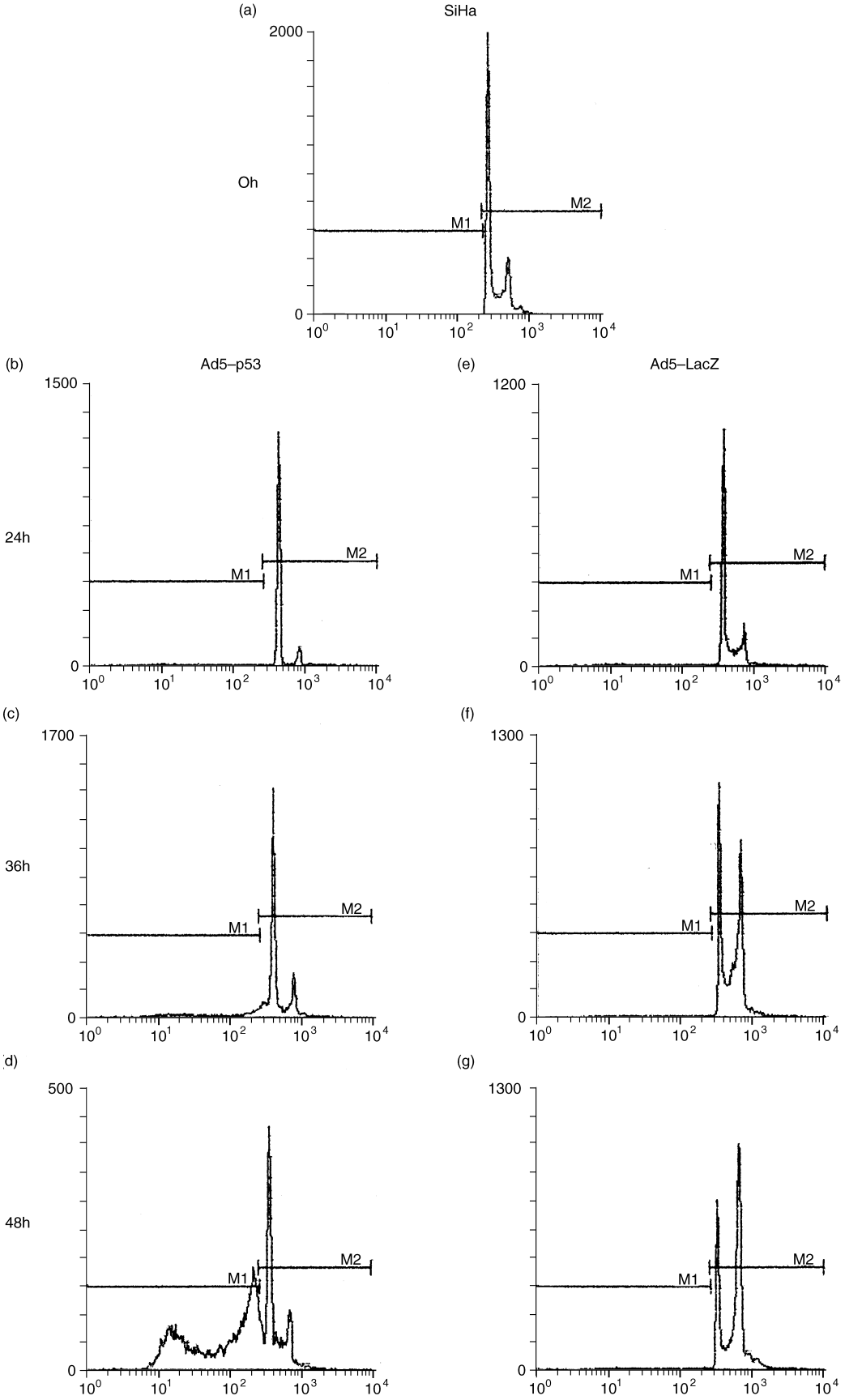

Fig. 3. The apoptosis of SiHa cells analysed with flow cytometry at 0 h (a), 24 h (b, e), 36 h (c, f) and 48 h (d, g) after transfection with Ad5-*p53* (b, c, d) at a MOI of 20. Transfections with the virus control Ad5-*LacZ* are shown on the right (e, f, g). M1, sub-G1 phase; M2, G1 phase.

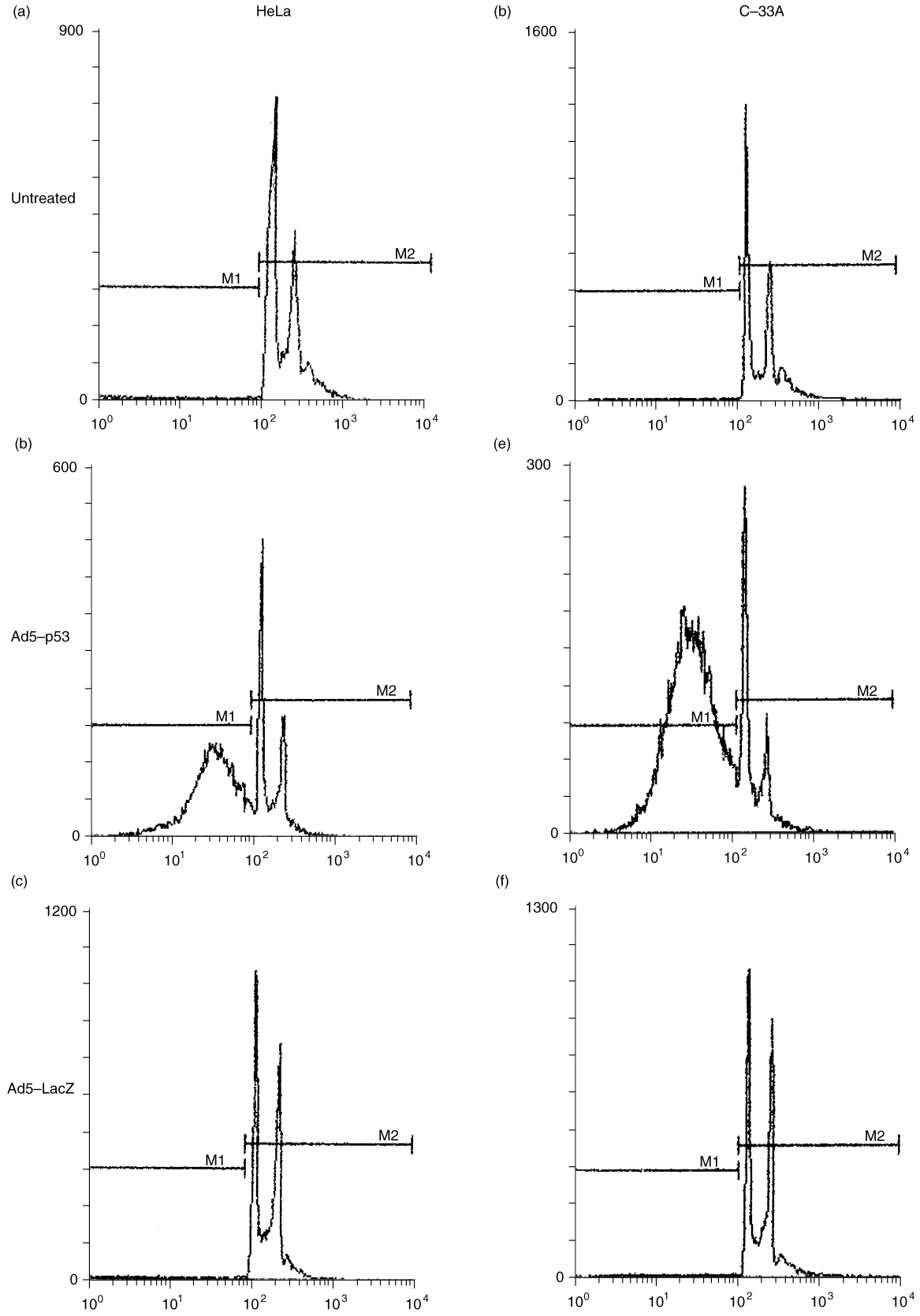

Fig. 4. The amount of apoptosis determined by flow cytometric analysis occurring in HeLa (a, b, c) at a MOI of 200; and C-33A cells (d, e, f) at a MOI of 50, 24 h after transfection with Ad5-*p53* (b, e), the untreated control (a, d) and the virus control Ad5-*LacZ* (c, f). M1 and M2 as above.

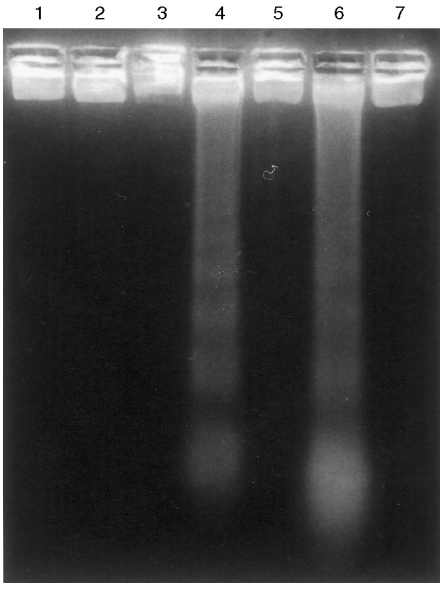

Fig. 5. SiHa cell DNA fragmentation analysed by agarose gel electrophoresis 24 h (lanes 2, 3), 36 h (lanes 4, 5) and 48 h (lanes 6, 7) after Ad5-*p53* transfection at a MOI of 20 (lanes 2, 4, 6), the cells (including detached cells) were harvested and treated with RNase. Untreated control (lane 1), and viral control (lanes 3, 5, 7).

SK-MEL-110 cells with a small $p21^{WAF1}$ induction, whilst Ad5-*p53* induced a moderate inhibition of growth of primary vascular smooth muscle cells with a high $p21^{WAF1}$ induction. Furthermore, the ectopically expressed $p21^{WAF1}$ protected SK-MEL-110 cells from p53-mediated apoptosis [22].

$p21^{WAF1}$, a downstream molecule of p53, is known to be upregulated by p53 [8,23]. In this study, the change of $p21^{WAF1}$ in the protein level did not run in parallel to the increase of p53 upon Ad5-*p53* transfection in HeLa and C-33A cells. On the contrary, $p21^{WAF1}$ was found to be decreased when high p53 was detected. We propose that the decrease in the $p21^{WAF1}$ protein level is related to apoptosis. This hypothesis is supported by results of the time course study of the cell cycle in SiHa cells, our data showed that the initial increase of $p21^{WAF1}$ corresponded to a G1 arrest at 24 h after transfection with Ad5-*p53*, thereafter, $p21^{WAF1}$ decreased, and the decrease was accompanied by an increase of apoptosis. Similarly, the decrease in $p21^{WAF1}$ protein level was associated with apoptosis in HeLa and C-33A cell lines when a higher MOI was used to transfect cells. Furthermore, in HeLa and C-33A cells, a small 14 kDa band reactive to the $p21^{WAF1}$ antibody was detected. This suggested that this smaller band of 14 kDa might be cleaved $p21^{WAF1}$. This observation is consistent with a recent report that $p21^{WAF1}$ is cleaved by caspases to create a p14 band in the early stages of the apoptotic pathway [24]. The reason why the 14 kDa band did not appear in SiHa cells is not clear. Another possible explanation for the decrease of $p21^{WAF1}$ in the presence of increasing p53 is a faster decay of $p21^{WAF1}$ at a high p53 level, leading to a predominance of apoptosis.

pRb, the master regulator of cell cycle, is physiologically regulated by p53 through $p21^{WAF1}$ and the cdks, and though the subsequent modulation of E2F activity [25]. pRb has been reported to cause growth arrest and inhibit apoptosis [3]. In this study, pRb phosphorylation was found to be lowered upon Ad5-*p53* transfection. Smaller bands of less than 110 kDa were detected, and this result is consistent with a reported study that an ICE-like proteinase can cause the proteolytic cleavage of pRb in the apoptotic process [26]. At the same time, Mdm-2, the p53 autoregulator, was found to be upregulated and cleaved upon Ad5-*p53* transfection in SiHa cells. Smaller bands of Mdm-2 were detected prior to the detection of any apoptosis, and Mdm-2 seems to decrease with time after Ad5-*p53* transfection. These results suggest that Mdm-2 could also be cleaved and/or decrease at a very early stage during the p53-dependent apoptosis. This observation is also in line with a previous report that Mdm-2 is cleaved by caspases during apoptosis [27]. Bax, which is upregulated by p53, has previously been shown to not be absolutely necessary for p53-dependent apoptosis. Bax mRNA and/or protein levels are not increased after p53 induction [12,13], and Bax-deficient cells are able to commit p53-dependent apoptosis upon irradiation [28]. In this study, Bax and Bcl-2 were found not to be induced by Ad5-*p53* in all three cervical cancer cell lines studied. Our data, together with those reported by others, suggest that p53, under our supraphysiological high condition, acts in the same way as in the physiological condition, i.e. p53 induces Mdm-2 and $p21^{WAF1}$, and $p21^{WAF1}$ inhibits the phosphorylation of pRb through the cyclin-dependent kinases [8]. However, p53 does not upregulate both Bax and Bcl-2.

The cause–result relationship between cell cycle status and p53-regulated proteins including pro-apoptotic factor Bax, anti-apoptotic factors $p21^{WAF1}$, pRb and Bcl-2 as well as Mdm-2, remains uncertain, and our results showed that the high $p21^{WAF1}$ was accompanied with G1 arrest whilst the decrease of $p21^{WAF1}$ was associated with increased apoptosis in SiHa cells. In addition, the data obtained also showed that anti-apoptotic molecules such as pRb and Mdm-2 were decreased or cleaved during apoptosis. These findings suggest that a decrease in $p21^{WAF1}$ expression may facilitate apoptosis in these cells. At the same time, the decrease and/or cleavage of pRb and Mdm-2 may also play roles in apoptosis. However, Bax and Bcl-2 were not found to be increased after Ad5-*p53* transfection in all three cell lines tested. Based on these observations, it seems that Bax and Bcl-2 are not important in p53-dependent apoptosis in these three cervical cancer cell lines.

Acknowledgements

We thank Professor J.P. Tao of the Beijing Medical University for his assistance in flow cytometric analysis. This study was funded by the Michael and Betty Kadoorie Foundation.

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