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Accumulation of p53 in response to adenovirus early region 1A sensitizes human cells to tumor necrosis factor alpha-induced apoptosis

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Abstract

Many tumor cells are resistant to tumor necrosis factor alpha (TNFα)-induced apoptosis. Adenovirus early region 1A (AdE1A) sensitizes the otherwise resistant cells to TNFα. AdE1A also stabilizes the p53 protein. The present study demonstrates a correlation between AdE1A-induced sensitization and stabilization of p53 in TNFα-induced apoptosis since the N-terminal and CR2 regions, the binding sites for CBP/p300, Rb and 26S proteasome regulatory components, are required for both these actions of AdE1A. TNFα does not induce apoptosis and AdE1A fails to sensitize TNFα cytotoxicity in p53-negative cells. However, introduction of exogenous p53 overcomes the cellular resistance to TNFα toxicity and enhances AdE1A sensitization, demonstrating that AdE1A sensitizes TNFα-induced apoptosis by its stabilization of p53. A proteasome inhibitor, lactacystin, enhances TNFα cytotoxicity in p53-positive and -negative cells, suggesting that accumulation of cellular proteins other than p53 might also regulate the cellular response to TNFα signaling.

Keywords: Adenovirus; AdE1A; TNFα; p53; Apoptosis; 26S proteasome; CBP/p300; Rb

Introduction

Adenovirus early region 1A (AdE1A) is the first protein to be expressed following viral infection and can generate diverse responses in host cells (Gallimore and Turnell, 2001; Frisch and Mymryk, 2002). It initiates cell cycle progression, driving quiescent cells into S phase, inhibits differentiation, and can induce apoptosis or cell immortalization. AdE1A produces these effects in the target cell by binding to a large number of cellular proteins (Gallimore and Turnell, 2001; Endter and Dobner, 2004). Two major products, referred to as 12S and 13S, are expressed and four highly conserved regions (CR) identified in AdE1A from different viral serotypes (Kimelman et al., 1985; Avvakumov et al., 2002, 2004). In Ad2/5, CR1 extends from residues 41 to 80 and CR2 from 121 to 139, while CR3 is the region unique to the larger 13S AdE1A and CR4 encompasses the C terminal region. These conserved regions can serve as binding sites for multiple cellular proteins. For example, the retinoblastoma (Rb) family of tumor suppressor proteins binds to CR1 and CR2 (Whyte et al., 1988; Jelsma et al., 1989). CR2 is also required for AdE1A's interaction with the S2 non-ATPase regulatory subunit of the 26S proteasome (Zhang et al., 2004). Components of the general and specific transcriptional machinery (e.g., TBP and ATF2) bind to the 13S-specific CR3 domain (Geisberg et al., 1994; Liu and Green, 1994), and the C terminus of AdE1A (CR4) targets C-terminal binding protein (CtBP) (Boyd et al., 1993; Chinnadurai, 2002, 2004). However, many cellular regulatory proteins interact through the less highly conserved N-terminal region. AdE1A can repress transcription of target genes by binding to CREB-binding protein (CBP) and its homologue p300 through the N-terminal region and the C-terminal half of CR1 (Eckner et al., 1994; Brockmann and Esche, 2003). In

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addition, TBP (Song et al., 1997), p400 (Fuchs et al., 2001), and the S4 and S8 ATPase regulatory components of the 26S proteasome (Turnell et al., 2000; Zhang et al., 2004) have been shown to bind close to the N-terminus. In vivo, it appears that both the N-terminal region and CR2 are involved in AdE1A interaction with the 26S proteasome (Zhang et al., 2004).

AdE1A stabilizes the tumor suppressor protein p53 by increasing its half-life (Braithwaite et al., 1990; Lowe and Ruley, 1993; Querido et al., 1997). Accumulation of p53 induced by AdE1A can cause apoptosis under different conditions (Debbas and White, 1993; Querido et al., 1997). Additionally, AdE1A can trigger apoptosis through p53-independent pathways (Chiou and White, 1997; Putzer et al., 2000). So far, at least three mechanisms explaining AdE1A-induced stabilization of p53 have been proposed. Firstly, p53 protein is stabilized due to AdE1A binding to CBP/p300 which regulates p53 degradation (Querido et al., 1997; Somasundaram and El-Deiry, 1997; Grossman, 2001). CBP/p300 promotes p53 degradation that is mediated by the ubiquitin ligase Mdm2 (Thomas and White, 1998; Kawai et al., 2001). It has been shown that CBP itself possesses an intrinsic ubiquitin ligase activity, directly inducing poly-ubiquitination of p53 (Grossman et al., 2003). Secondly, Rb might play a role in the regulation of p53 stability in human cells (Querido et al., 1997). Inactivation of Rb by AdE1A induces accumulation of $p19^{ARF}$ that in turn induces p53 accumulation (de Stanchina et al., 1998; Samuelson et al., 2005). Thirdly, p53 stabilization can be induced by AdE1A's specific interaction with, and inhibition of, the 26S proteasome through the N-terminal and CR2 regions (Grand et al., 1999; Turnell et al., 2000; Zhang et al., 2004). Notably, AdE1A mutants lacking capacity to interact with the 26S proteasome fail to stabilize p53.

Tumor necrosis factor-alpha (TNFα) is a multi-functional cytokine that plays a critical role in controlling apoptosis in many malignant cell types (Yagita et al., 2004; Gupta and Gollapudi, 2005). It exerts cytotoxic effects on some cells, but many are resistant to it. It has been known for some time that AdE1A can sensitize otherwise resistant cells to TNFα-induced apoptosis (Chen et al., 1987; Duerksen-Hughes et al., 1989). In mouse fibroblasts, for example, the domains of AdE1A responsible for inducing susceptibility to TNFα involve N-terminal and/or CR2 regions which are the binding sites for CBP/p300 and Rb (Shisler et al., 1996; Cook et al., 2002). It has been suggested that CBP/p300 functions with NFκB to protect against TNFα cytotoxicity (Vanden Berghe et al., 1999). AdE1A represses the TNFα-induced NFκB activation response through interaction with both CBP/p300 and Rb (Shao et al., 1999) or Rb alone (Cook et al., 2002). It has been shown that TNFα-induced apoptosis is a p53-dependent cellular response (Jeoung et al., 1995). Indeed, restoration of wild-type p53 function in p53 mutant cells increases their susceptibility to the cytotoxic action of TNFα (Ameyar-Zazoua et al., 2002). However, TNFα-induced apoptosis can also occur independently of p53 in human and mouse cells (Yoshida et al., 1996; Cook et al., 1999).

Although increasing lines of evidence suggest that the N-terminal and CR2 domains in AdE1A are essential for interaction with CBP/p300, Rb, and the regulatory components of the 26S proteasome and subsequent inhibition of p53 degradation, the requirement for expression of p53 and binding to these proteins in AdE1A sensitization to TNFα-induced apoptosis is still poorly understood. We set out, in the present study, to investigate the relationship between these two activities in TNFα-induced apoptosis in human cells. The results presented here demonstrate that expression of p53 enhances TNFα-induced apoptosis and that AdE1A sensitizes otherwise resistant cells to TNFα toxicity by stabilizing p53 through the N-terminal and CR2 regions.

Results

Both the N-terminal and CR2 regions of AdE1A are required to sensitize A549 cells to TNFα-induced apoptosis

Experiments in mouse fibroblasts have shown that mutant viruses with deletions in the N-terminal and CR2 regions of AdE1A were unable to induce sensitization to TNFα-induced apoptosis (Shisler et al., 1996). Another study reported that a deletion of CR2 alone was sufficient to abrogate AdE1A sensitization in the same cells (Cook et al., 2002). To determine the domains of AdE1A responsible for the sensitization of human tumor cells to TNFα cytotoxicity, we tested a number of AdE1A mutants.

Firstly, the AdE1A sensitization to the cytotoxicity of TNFα was examined in non-small cell human lung carcinoma cells (A549). Ad5 *dl*520 virus, which expresses 12S AdE1A but does not express 13S AdE1A, E1B 19K, E1B 55K, and E3 proteins, was used for infection studies since E1B and E3 genes possess anti-apoptotic activities (Gooding et al., 1988; Wold, 1993; Burgert et al., 2002). A549 cells were infected with Ad5 *dl*520 virus for 24 h then treated with TNFα. The results show that approximately 29% of cells infected with Ad5 *dl*520 in the presence of TNFα were apoptotic at 24 h, while less than 5% of cell death was observed in samples treated with TNFα or the virus alone (Fig. 1). These results demonstrate that A549 cells were initially resistant to the cytotoxicity of TNFα and became sensitized by the expression of AdE1A. The synergistic induction of apoptosis in A549 cells provided a good model for the further studies on AdE1A sensitization to TNFα.

Next, a panel of Ad5 mutant viruses derived from Ad5 *dl*520 with various deletions in AdE1A was used to infect A549 cells. *dl*1101 and *dl*0106 had a deletion of amino acids 4–25 at the N-terminus of AdE1A, and the latter had additional deletions in the region between CR1 and CR2; *dl*1108 had a deletion in the CR2 region, while *dl*0108 had a

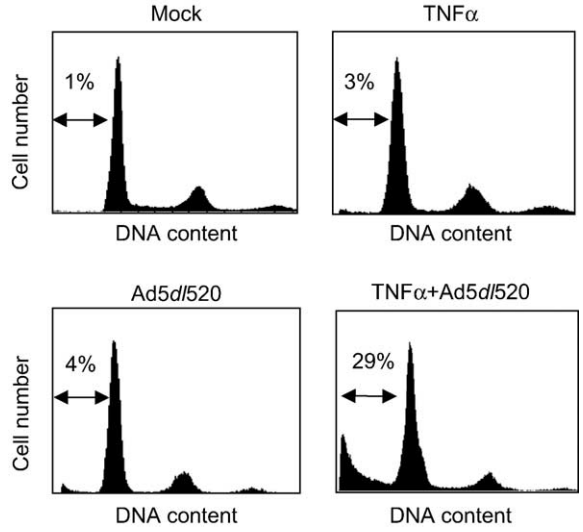


Fig. 1. AdE1A sensitizes adenovirus-infected A549 cells to TNFα-induced apoptosis. A549 cells were infected with adenovirus Ad5*dl*520 (50 pfu/cell). 24 h post-infection, the cells were treated with or without TNFα (20 ng/ml) for a further 24 h, followed by fixation with ethanol and staining with propidium iodide. The percentage of cytotoxicity was determined by measuring DNA content of the sub-G1 population in stained cells using FACS analysis. Uninfected cells were regarded as "mock".

double deletion in both the N-terminal region and CR2 (Fig. 2A). After 24 h infection, cells were treated with TNFα for a further 24 h prior to being photographed under phase-contrast microscopy. It appeared that, like *dl*520, the mutants *dl*1101, *dl*0106, and *dl*1108 enhanced cell sensitivity to TNFα, but the double mutant *dl*0108 did not (Fig. 2B). Fig. 2C shows that the percentage of TNFα-induced apoptosis produced by *dl*0107 and *dl*0108 was much less (<10%) than that by the mutants *dl*520, *dl*1101, *dl*0106, *dl*1107, or *dl*1108 (26–36%). The differences between *dl*0107 or *dl*0108 and other mutants are statistically significant ($^{\#}P < 0.01$). These findings are supported by cell proliferation studies determined by alamarBlue assays. Unlike *dl*520, *dl*1101, *dl*0106, *dl*1107, and *dl*1108, the double mutant *dl*0107 or *dl*0108 did not induce significant growth inhibition (Fig. 2D). These results demonstrate that AdE1A sensitization is largely dependent on the amino acid sequences in the N-terminal region and CR2 of AdE1A since the viruses with deletions in these sites (*dl*0107 and *dl*0108) failed to sensitize cells to TNFα-induced apoptosis. Deletion of either the N-terminal region (*dl*1101) or CR2 (*dl*1107 and *dl*1108) alone did not abrogate AdE1A sensitization.

Adenovirus expressing 12S AdE1A only can replicate under certain conditions during viral infection (Winberg and Shenk, 1984). AdE1A mutants deleted in the N-terminal and CR2 regions have been shown to prevent S-phase induction and viral replication in tumor cells (Howe and Bayley, 1992; Shisler et al., 1996; Somasundaram and El-Deiry, 1997; Sauthoff et al., 2004). To test if the sensitization induced by adenoviruses is a direct result of expression of AdE1A proteins or a general response to viral replication, the levels of AdE1A, along with the structural proteins hexon and penton, were examined in A549 cells infected by various viruses for 48 h. AdE1A was expressed at similar levels in the cells infected by all the mutants. Viral structural proteins were also expressed in all of the infected samples although the levels in the cells infected with double mutant *dl*0107 or *dl*0108 were slightly reduced (Fig. 2E). This result indicates that expression of AdE1A with deletions in the N-terminus and CR2 region may restrict the rate of viral replication, which might, in turn, further inhibit the capacity of the mutant viruses in the sensitization of TNFα cytotoxicity as suggested by others (Sauthoff et al., 2004).

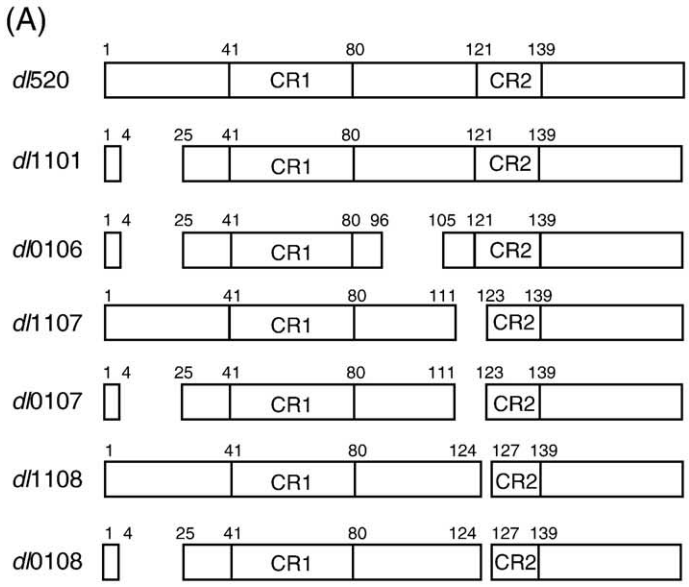
The ability of AdE1A to sensitize cells to TNFα-induced apoptosis correlates with its ability to stabilize p53

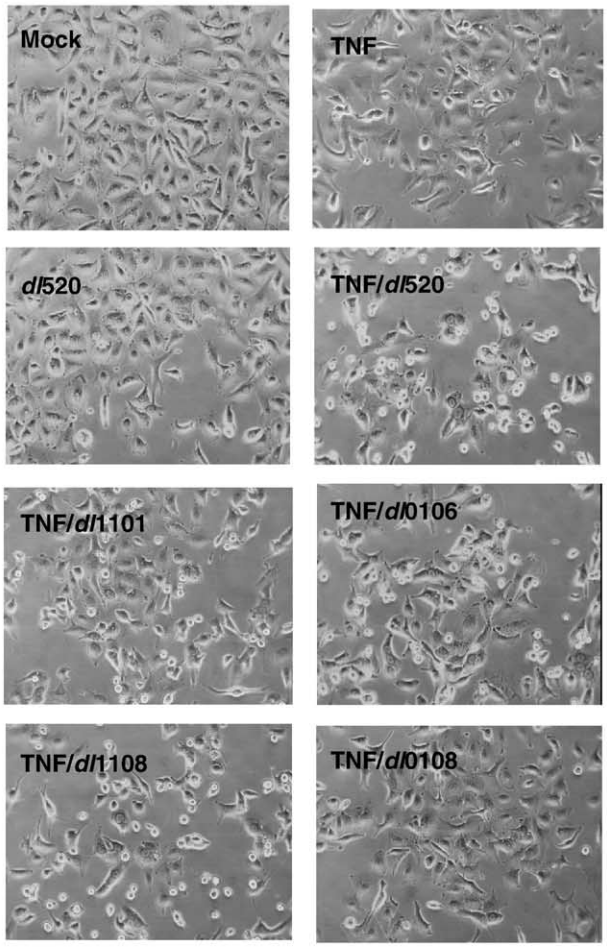
Although it is well established that AdE1A can sensitize cells to TNFα cytotoxicity and stabilize the p53 protein, the relationship between these two activities in TNFα-treated cells is not known. For this reason, we examined the regions involved in both AdE1A sensitization and p53 stabilization in human cells treated with TNFα. Since the mutant viruses used in this study would produce other adenoviral proteins such as E4Orf6 that might contribute to the effect of AdE1A (Debbas and White, 1993; Moore et al., 1996), transient transfection was used to express AdE1A in A549 cells. Our results show that wild-type 12S AdE1A dramatically enhanced TNFα-induced apoptosis ($^{\#}P < 0.01$), although it did not induce marked cell death at 24 h on its own (Fig. 3A). Similar to the results from viral infection, expression of AdE1A from a double mutant containing deletions in the N-terminal and CR2 regions (*dl*0108) failed to sensitize cells to TNFα cytotoxicity. This result confirms that expression of AdE1A protein alone directly induces the sensitization to TNFα-induced cytotoxicity, and actual viral replication plays only a minor role in the process.

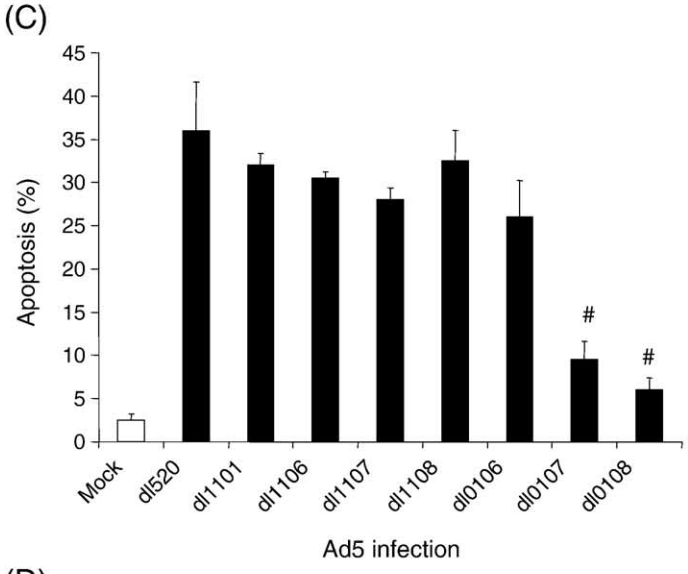
Accordingly, p53 levels in the transfected cells were elevated by *wt* AdE1A but not by *dl*0108, while AdE1A proteins were expressed at comparable levels (Fig. 3B). Interestingly, the increase in p53 levels by AdE1A was not accompanied by an increase of apoptosis in the absence of TNFα. The enhancement of apoptosis by AdE1A was observed only in cells treated with TNFα. The observation that deletion of the N-terminal and CR2 regions abrogated both AdE1A sensitization and stabilization of p53 in TNFα-treated A549 cells clearly demonstrates that the accumulation of p53 by AdE1A correlates closely to AdE1A sensitization to TNFα cytotoxicity.

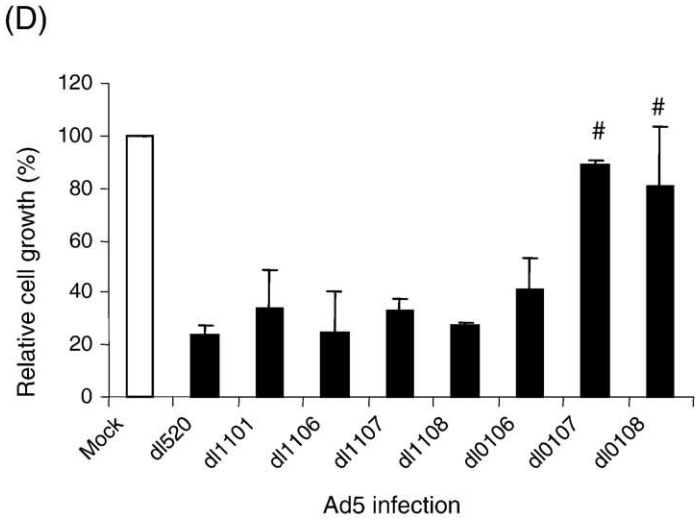
Induction of p53 overcomes cellular resistance to TNFα toxicity

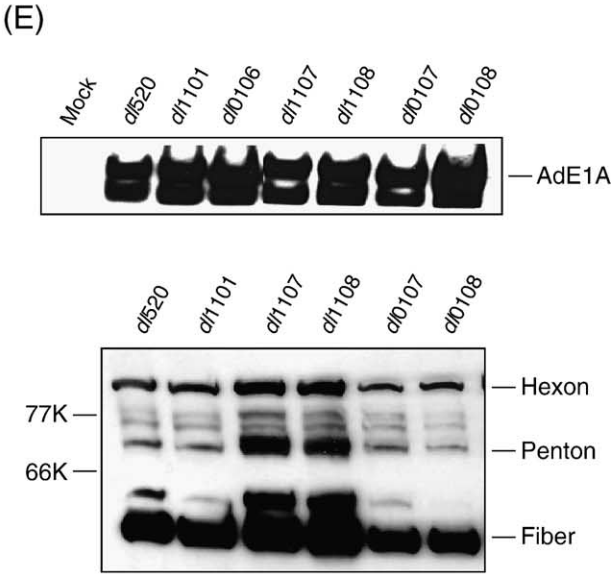
The results shown above suggest that the accumulation of p53 might be critical in the regulation of the cellular


(A)
dl520
dl1101
dl0106
dl1107
dl0107
dl1108
dl0108
CR1
CR2


(B)
Mock
TNF
dl520
TNF/dl520
TNF/dl1101
TNF/dl0106
TNF/dl1108
TNF/dl0108


(C)
Apoptosis (%)
Mock
dl520
dl1101
dl1106
dl1107
dl1108
dl0106
dl0107
dl0108
Ad5 infection


(D)
Relative cell growth (%)
Mock
dl520
dl1101
dl1106
dl1107
dl1108
dl0106
dl0107
dl0108
Ad5 infection


(E)
Mock
dl520
dl1101
dl0106
dl1107
dl1108
dl0107
dl0108
AdE1A
77K
66K
Hexon
Penton
Fiber

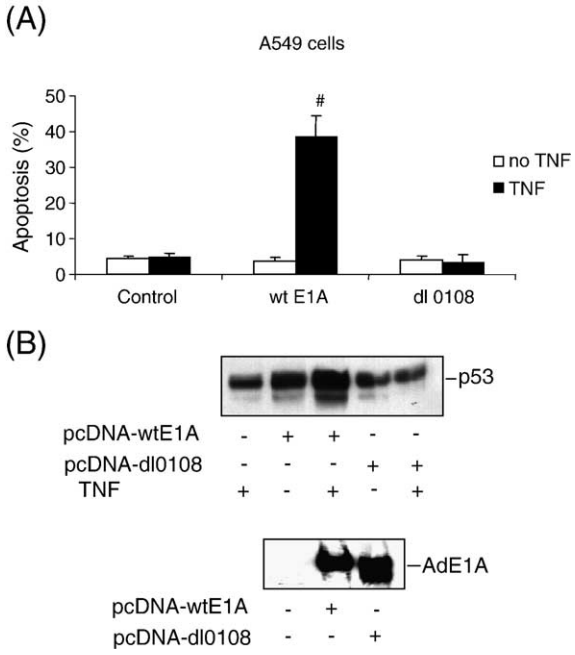


Fig. 3. AdE1A sensitization in TNFα-induced apoptosis correlates to its ability to stabilize p53 protein in transient transfected A549 cells. A549 cells were transfected with 4 μg of pcDNA3-*wt*AdE1A plasmid which expresses wild-type 12S, or with 4 μg of pcDNA3-*dl*0108 plasmid with deletions in N-terminal and CR2 regions of AdE1A. After 24 h, cells were treated with 20 ng/ml of TNFα for a further 24 h. The cells were then harvested and subjected to apoptosis analysis by FACS assays (A). Empty vector pcDNA3 was used as control plasmid. Data were mean values of duplicates from two independent experiments. $^{\#}P < 0.01$ when compared with cells treated with TNFα alone. Expression of p53 and AdE1A proteins were detected by Western blot analysis (B).

response to TNFα toxicity. We examined this possibility further by transfection of pcDNA-p53 plasmid into H1299 cells that do not express endogenous p53 protein. H1299 cells were found to be resistant to TNFα cytotoxicity but became sensitized following exogenous expression of p53 (Fig. 4A, $^{\#}P < 0.01$), indicating that p53 can overcome cellular resistance to TNFα toxicity. Expression of p53 itself did not induce apoptosis. In addition, we examined whether increased expression of p53 would enhance TNFα cytotoxicity in p53-expressing cells. A549 cells were initially resistant to TNFα toxicity. Over-expression of p53, shown as elevated p53 levels in cells transfected with pcDNA-p53, did not increase apoptosis in the absence of TNFα. In the presence of TNFα, however, a significant increase in the percentage of apoptotic cells was seen in the cells where p53 was over-expressed (Fig. 4B, $^{\#}P < 0.01$). In contrast to the observation by others (Kim et al., 2002), we found that TNFα did not increase p53 level. The results suggest that p53 protein could serve as a cause, but not a marker, of enhanced cellular susceptibility to TNFα-induced apoptosis, although induction of p53 itself need not be sufficient to trigger apoptosis.

Expression of p53 enhances AdE1A sensitization to TNFα-induced apoptosis

Subsequently, we examined whether expression of p53 can enhance AdE1A sensitization to TNFα-induced apoptosis. In p53-positive A549 cells, AdE1A stabilized p53 in cells treated with or without TNFα, but apoptosis was observed only in the presence of both AdE1A and TNFα. In addition, additive accumulation of p53 by co-expression of AdE1A and p53 did not increase apoptosis in the absence of TNFα (Fig. 5A). However, an increase in the percentage of apoptosis by co-expression of p53 and AdE1A was observed in cells treated with TNFα (57%), when compared to the samples treated with only AdE1A and TNFα (39%). This indicates that over-expression of p53 was responsible for the additional 18% increase in apoptosis observed in the AdE1A sensitization ($^{\#}P < 0.01$). Moreover, with no endogenous p53, H1299 cells were resistant to TNFα and AdE1A sensitization (Fig. 5B). In the absence of TNFα, expression of exogenous p53 and AdE1A did not significantly increase apoptosis when compared with cells expressing AdE1A alone ($^{*}P > 0.05$). However, expression of p53 protein in H1299 cells significantly enhanced AdE1A sensitization to TNFα cytotoxicity ($^{\#}P < 0.01$). The expression of p53 and AdE1A proteins was detected by Western blot analysis. Based on the observation that expression of p53 protein increased cell susceptibility to TNFα toxicity and enhanced AdE1A sensitization, we conclude that the accumulation of p53 protein is, at least partly, responsible for AdE1A sensitization to TNFα-induced apoptosis.

The proteasome inhibitor lactacystin promotes TNFα-induced apoptosis regardless of expression of p53 and AdE1A

Our data suggested that AdE1A-induced sensitization to TNFα cytotoxicity is related to the ability of AdE1A to inhibit the activity of the 26S proteasome and the

Fig. 2. AdE1A sensitizes TNFα-induced cell killing through its N-terminal and CR2 regions in adenovirus infected A549 cells. A549 cells were infected with mutant adenoviruses derived from Ad5*dl*520. The regions of deletions in the AdE1A gene are shown in panel A. A549 cells were infected with Ad5 mutant viruses (50 pfu/cell) for 24 h. After infection, the cells were treated with TNFα for 24 h prior to being photographed under phase-contrast microscopy using ScionImage software (B). The infected cells were stained with acridine orange fluorescent dye. The percentage of apoptotic cells were counted under the microscope (C). A549 cells were cultured in 96-well plates and infected with Ad5 mutant viruses (50 pfu/cell) for 24 h. The cells were then treated with TNFα (20 ng/ml) for a further 24 h and stained with alamarBlue dye at 37 °C for 4 h for color development (D). The results were mean values of duplicates (C) or triplicates (D) from two independent experiments. $^{\#}P < 0.01$ when compared to the samples treated with other mutant viruses. Western blots showing expression of AdE1A and viral structural proteins after infection for 48 h are presented in panel E.

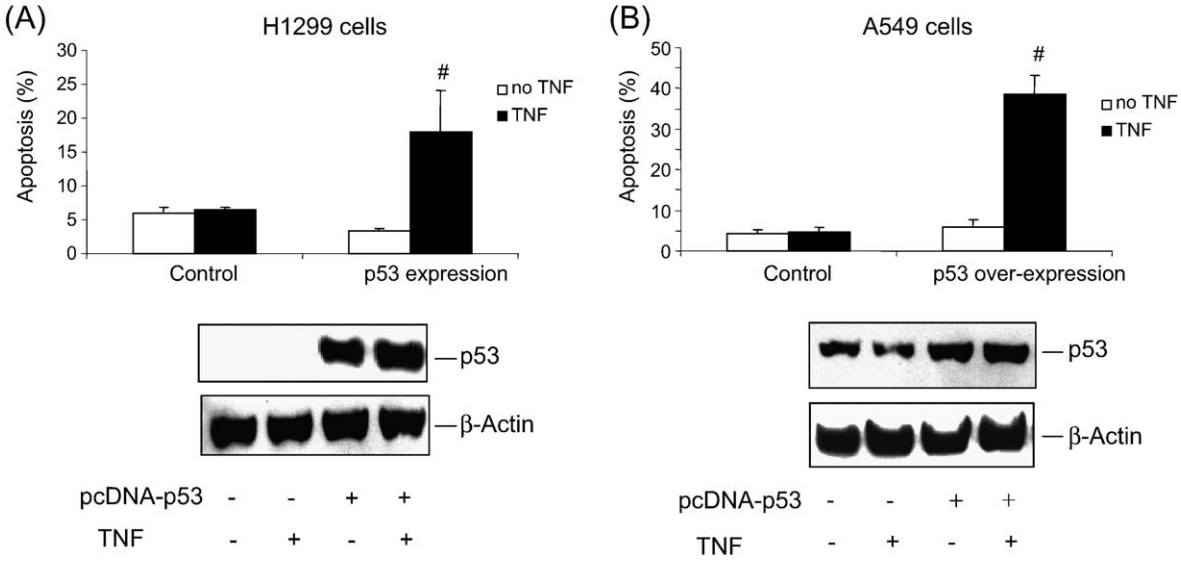


Fig. 4. Induction of p53 protein increases cell susceptibility to TNFα-induced apoptosis and AdE1A sensitization. (A) H1299 cells were transfected with 0.5 μg of pcDNA3-p53 for 24 h, followed by treatment with 20 ng/ml of TNFα for a further 24 h. (B) A549 cells were transfected with 2 μg of pcDNA3-p53 for 24 h, followed by treatment with 20 ng/ml of TNFα for a further 24 h. The cells were then harvested and subjected to apoptosis analysis by FACS assays. Empty vector pcDNA3 was used as control plasmid. Data were mean values of duplicates from two independent experiments. $^{\#}P < 0.01$ when compared with cells treated with TNFα alone. Expression of p53 and AdE1A proteins were detected by Western blot analysis.

degradation of p53. For this reason, we investigated the effect of the proteasome inhibitor lactacystin on TNFα-treated cells. Incubation with lactacystin causes accumulation of polyubiquitinated proteins, including p53, in apoptotic cells (Lopes et al., 1997). p53-positive (A549) and p53-negative (H1299) cells were treated with lacta-

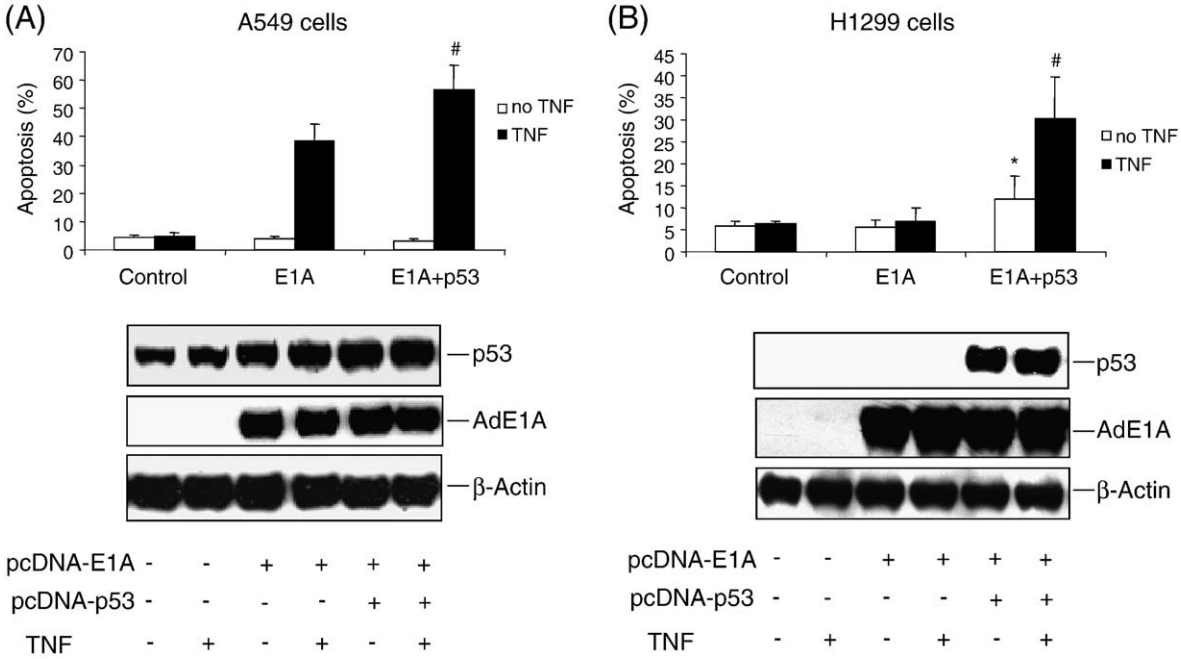


Fig. 5. Expression of exogenous p53 proteins renders H1299 cells susceptible to TNFα-induced apoptosis and AdE1A sensitization. (A) A549 cells were co-transfected with 2 μg of pcDNA3-p53 and 4 μg of pcDNA3-12S AdE1A for 24 h, followed by treatment with 20 ng/ml of TNFα for a further 24 h. $^{\#}P < 0.01$ when compared with cells treated with AdE1A and TNFα. (B) H1299 cells were co-transfected with 0.5 μg of pcDNA3-p53 and 1 μg of pcDNA3-12S AdE1A for 24 h, followed by treatment with 20 ng/ml of TNFα for a further 24 h. The cells were then harvested and subjected to apoptosis analysis by FACS assays. Data were mean values of duplicates from two independent experiments. $^{\#}P < 0.01$ when compared with cells treated with AdE1A and TNFα. $^{*}P > 0.05$ when compared with cells treated with AdE1A alone. Empty vector pcDNA3 was used as control plasmid. Expression of p53 and AdE1A proteins were detected by Western blot analysis.

cystin, in the absence or the presence of TNFα. The results show that lactacystin enhanced TNFα-induced apoptosis in both p53-negative and -positive cells (Fig. 6). This indicates that TNFα could induce apoptosis through p53-dependent and -independent pathways and that the accumulation of other cellular proteins as a result of proteasomal inhibition could also sensitize cells to TNFα toxicity.

We have previously shown that a major mechanism underlying p53 stabilization by AdE1A is the inhibition of proteasome activity through the interaction of AdE1A with the 19S regulatory components of the 26S proteasome (Turnell et al., 2000; Zhang et al., 2004). In the present study, we have compared the effects of accumulation of p53 protein induced by AdE1A and lactacystin on the cellular response to TNFα toxicity. A549 cells were transfected with pcDNA3-12SAdE1A plasmid then treated with lactacystin and TNFα (Fig. 7A). In the absence of AdE1A, an additive effect was observed in cells treated with both TNFα and lactacystin. While TNFα-induced apoptosis was enhanced by the expression of AdE1A, addition of lactacystin further increased the AdE1A sensitization.

The effect of lactacystin on cell proliferation was also evaluated (Fig. 7B). A549 cells were infected with Ad5 *dl*520 virus and then treated with lactacystin, in the absence or the presence of TNFα. The data show that lactacystin increased the inhibition of cell growth induced by AdE1A and TNFα. Collectively, these data demonstrate an additive effect of lactacystin on AdE1A sensitization in TNFα-induced apoptosis and growth arrest.

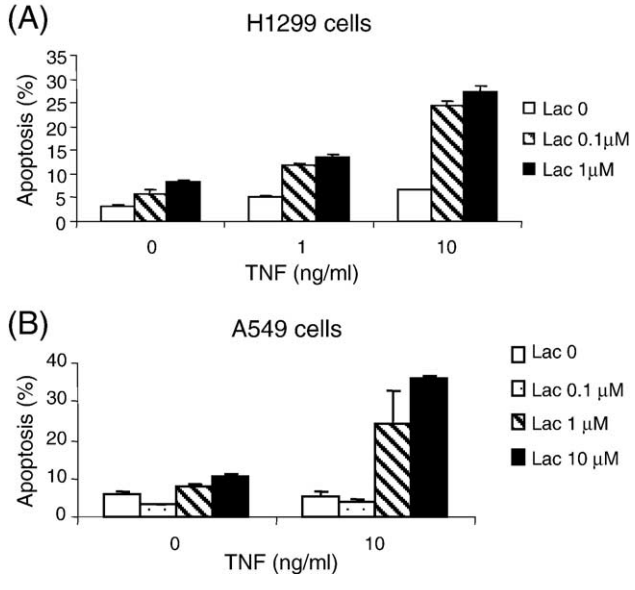


Fig. 6. The proteasome inhibitor lactacystin promotes TNFα-induced apoptosis in tumor cells independent of p53 status. p53-negative H1299 cells (A) and p53-positive A549 (B) cells were treated for 24 h with the proteasome inhibitor lactacystin (0.1, 1, or 10 μM), in the absence or the presence of TNFα at concentration of 1 or 10 ng/ml. Cells were subjected to apoptosis analysis by trypan blue exclusion assay. Data were mean values of duplicates from two independent experiments.

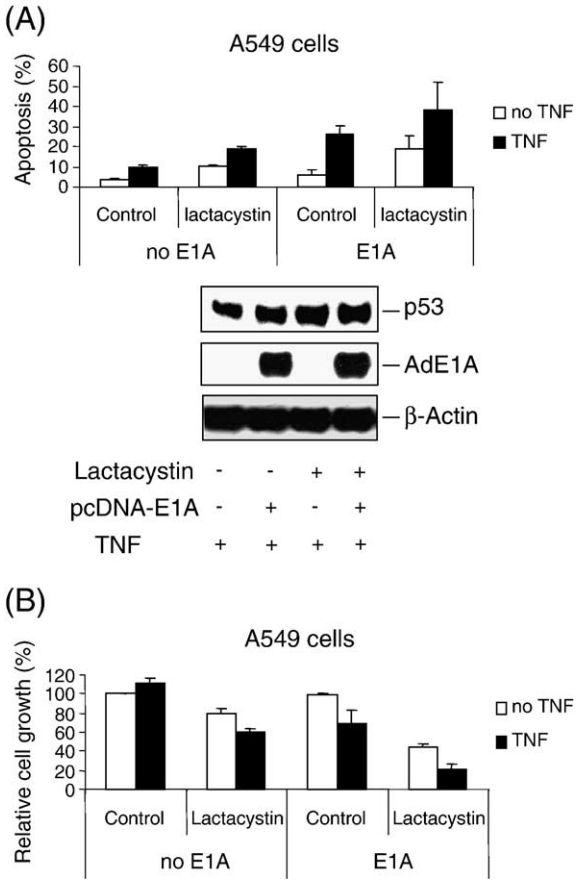


Fig. 7. Lactacystin enhances TNFα-induced apoptosis and inhibits cell growth in the absence or the presence of AdE1A. (A) A549 cells were transfected with pcDNA3-12SAdE1A plasmid for 24 h, prior to further 24 h incubation with lactacystin (10 μM), in the absence or the presence of 20 ng/ml of TNFα. Cells were subjected to apoptosis analysis by FACS assays. Expression of p53 and AdE1A proteins were detected by Western blot analysis. (B) A549 cells were cultured in 96-well plates and infected with Ad5*dl*520 (50 pfu/cell) for 24 h. Cells were then treated with lactacystin (10 μM), in the absence or the presence of TNFα (20 ng/ml) for 24 h, prior to cell proliferation analysis by alamarBlue assays. Data were mean values of duplicates or triplicates from two independent experiments.

Discussion

We have previously shown that AdE1A's interaction with the regulatory complex of the 26S proteasome in vivo requires the N-terminal region and CR2 (Turnell et al., 2000; Zhang et al., 2004). In vitro mutational analysis has revealed that AdE1A interacts with the S4 and S8 ATPase subunits through its N-terminal region and binds to the S2 non-ATPase subunit through CR2 region. Deleting both N-terminal and CR2 regions is needed for abrogation of AdE1A's interaction with the 26S proteasome in vivo. These interactions result in an inhibition of 26S proteasomal

activity, which causes the accumulation of p53 protein (Zhang et al., 2004).

In the present study, we have investigated the biological consequences of this interaction and established a correlation between AdE1A-induced sensitization to TNFα-induced apoptosis and stabilization of p53 in TNFα-treated human A549 cells. Stabilization of p53 and sensitization to TNFα-induced apoptosis required the N-terminal and CR2 regions of AdE1A that are also the binding sites for the 26S proteasome (Figs. 2 and 3). This suggests that the accumulation of p53 protein through inhibition of the 26S proteasome might serve as a novel mechanism for AdE1A in governing cell susceptibility to TNFα-induced apoptosis.

In p53-negative human H1299 cells, TNFα did not induce apoptosis and AdE1A failed to enhance TNFα cytotoxicity. However, expression of exogenous p53 overcame the cellular resistance to TNFα toxicity (Fig. 4). These results suggest that p53 plays a critical role in the TNFα signaling pathway and that high levels of p53 are sufficient for triggering TNFα-induced apoptosis. This is consistent with other reports that infection of TNFα-resistant MCF7 cells with Ad*wt*p53 restores cell sensitivity to TNFα toxicity (Ameyar et al., 1999; Shatrov et al., 2000). We also observed that over-expression of p53 additively enhanced AdE1A sensitization to TNFα-induced apoptosis in the otherwise resistant A549 cells, which endogenously express wild-type p53 protein (Fig. 5). Given the finding that induction of p53 overcame TNFα resistance and that AdE1A sensitization to TNFα-induced apoptosis was observed only when p53 was induced or stabilized, we conclude that accumulation of p53 protein in response to AdE1A is a key component of the mechanism by which AdE1A sensitizes human cells to TNFα.

Our observation that reintroduction of p53 protein in p53-negative cells restored cell susceptibility to TNFα toxicity suggests that p53 can function as a key regulator, governing the cellular response to TNFα. However, it does not exclude the possibility that other cellular proteins are involved. In the present study, the requirement for p53 in the regulation of TNFα resistance was further evaluated using the proteasome inhibitor lactacystin. This drug inhibits the activity of the 20S proteasome complex and prevents the degradation of numerous regulatory proteins, including p53, resulting in an overload of conflicting signals in cancer cells (Fenteany and Schreiber, 1998). Many of these signals inhibit cell growth, eventually inducing apoptosis. Our data show that lactacystin induced apoptosis in cells independent of p53 status (Fig. 6), suggesting that the cell death can be induced as a result of increased intracellular concentrations of other regulatory proteins that are normally degraded by the 26S proteasome. Not surprisingly, while expression of p53 can overcome TNFα resistance and enhance TNFα-induced apoptosis, it is not the sole factor regulating the complex phenomenon of TNFα resistance and the sensitization to TNFα by AdE1A. For instance, it has been reported that AdE1A can sensitize cells to TNFα cytotoxicity by down-regulating c-FLIP and preventing the induction of c-FLIP by TNFα (Perez and White, 2003). Interestingly, additive effects on the level of TNFα-induced apoptosis were observed when A549 cells were treated with AdE1A and lactacystin (Fig. 7), suggesting that AdE1A and lactacystin could additively inhibit the 26S proteasomal activity through targeting the regulatory complex and the 20S proteasome, respectively.

Previous studies have suggested that the molecular mechanism underlying AdE1A sensitization to TNFα cytotoxicity can be attributed to the interaction of AdE1A with CBP/p300 and/or Rb, resulting in inhibition of NFκB (Shisler et al., 1996; Shao et al., 1999; Cook et al., 2002). Intriguingly, both CBP/p300 and Rb have been shown to regulate p53 expression levels. Although CBP/p300 functions as a co-activator of p53-dependent transcription, it also enhances p53 turnover by stabilizing its negative regulator, the Mdm2 ubiquitin ligase (Somasundaram and El-Deiry, 1997; Kawai et al., 2001; Grossman et al., 2003). Thus, AdE1A can stabilize p53 by sequestering CBP/p300 (Querido et al., 1997). The inactivation of Rb by AdE1A can also result in increased levels of $p14^{ARF}$ or $p19^{ARF}$, which then binds to and inactivates Mdm2, leading to a stabilization of p53 (Bates et al., 1998; Sherr and McCormick, 2002; Rocha et al., 2005). An indirect role for Rb in the regulation of p53 function has also been recently described. ASPP2 (53BP2L), a p53-binding protein that promotes apoptosis, has been shown to be an E2F target gene, indicating a role for Rb/E2F in regulating p53-dependent apoptosis (Chen et al., 2005). Therefore, AdE1A binding to Rb could free E2F to activate transcription of ASPP2, ultimately stimulating p53-dependent apoptosis. Further studies are needed to elucidate the role of Rb/E2F pathway in the regulation of p53 stability and TNFα-induced apoptosis.

Taking the existing literature and the results of the present study into account, the evidence suggests that targeting the 26S proteasome, CBP/p300, and Rb all probably contribute to the sensitization of cells to TNFα-induced apoptosis by AdE1A (Table 1). It seems possible that the particular role attributable to each of these AdE1A binding proteins could very likely depend on the cellular context. The precise contribution of the interactions with CBP/p300, Rb, and the 26S proteasome to the regulation of p53 stability and AdE1A sensitization in TNFα-induced apoptosis will be investigated in the future when AdE1A mutants with differential binding capacities for these proteins become available.

Taken together, the results from the present study demonstrate that inhibition of proteasomal degradation and subsequent accumulation of p53 is a novel mechanism by which AdE1A sensitizes human cells to TNFα-induced apoptosis. These observations also reveal that p53 plays a key role in the regulation of TNFα-induced apoptosis. AdE1A has already been utilized as a valuable tool in the studies of molecular mechanisms of TNFα signaling path-

Table 1
Properties of adenoviruses expressing mutant AdE1A

Ad5 viruses	Deletion positions	Binding CBP/p300	Binding Rb	Binding proteasome	p53 stabilization	TNFα sensitization
Ad5*dl*520	125 wt	+	+	+	+	+
Ad5*dl*1101	4–25	–	+	+	+	+
Ad5*dl*1106	96–105	+	+	+	+	+
Ad5*dl*1107	111–123	+	–	+	+	+
Ad5*dl*1108	124–127	+	–	+	+	+
Ad5*dl*0106	4–25, 96–105	–	+	+	+	+
Ad5*dl*0107	4–25, 111–123	–	–	–	–	–
Ad5*dl*0108	4–25, 124–127	–	–	–	–	–

Comparison of AdE1A's interaction with CBP/p300, Rb, and 26S proteasome with its ability to stabilize p53 and to sensitize TNFα-induced apoptosis is listed in this table. The findings for AdE1A's binding capacity for CBP/p300, Rb, and the 26S proteasome were documented previously.

way (Sang et al., 2002) and our studies demonstrate that AdE1A continues to be useful in unraveling the regulation of this complex response. Recent progress has been made towards the use of AdE1A-expressing adenovirus vectors in cancer therapy (Dobbelstein, 2004). Our findings may be useful in the development of new therapeutic strategies involving AdE1A in adenoviral vectors delivering TNFα and p53 genes for the treatment of human cancers.

Materials and methods

Cell lines and reagents

A549 is a non-small cell human lung carcinoma cell line expressing wild-type p53 and has been used for adenoviral infection experiments. H1299 cells are derived from a p53-null, non-small cell human lung carcinoma. Cells were grown in HEPES-buffered DME supplemented with 8% FCS and 2 mM glutamine. Human recombinant TNFα and proteasome inhibitor lactacystin were purchased from R & D Systems and Calbiochem, respectively.

Plasmids and transient transfection

A human pcDNA-p53 plasmid was kindly provided by Prof. David Lane (University of Dundee). Constructs containing Ad5 AdE1A mutants were described previously (Zhang et al., 2004). For transient transfection experiments, cells at 60–70% confluency were transfected with plasmids using LipofectAMINE 2000 reagent (Invitrogen). After 24 h, cells were harvested or given further treatment. The total amount of DNA used in each dish was kept constant using the corresponding empty vector, pcDNA3, which was also used in the control samples.

Viruses and viral infection

The adenovirus Ad5 *dl*520 used in this study is in the Ad5 *dl*309 background, which lacks the E3 domain encoding genes for E3 14.7K, 14.5K and 10.4K, but retains the wild-type AdE1A region (Jones and Shenk, 1979). The Ad5 *dl*520 mutants were created to express Ad5 *wt* or mutant 12SE1A, but not 13SE1A, E3, E1B 19K and E1B 55K proteins (Shepherd et al., 1993; Mymryk, 1998). The positions of AdE1A mutations in the Ad5 *dl*520 mutants are illustrated in Fig. 2A. For viral infection, sub-confluent A549 cells were infected with 12S *wt* (*dl*520) or mutant viruses in serum-free medium at 50 plaque forming units (pfu)/cell for 2 h at 37 °C, with intermittent rocking. Infected cells were then washed once and incubated in fresh complete medium for 24 h prior to further treatment with TNFα and/or lactacystin for detection of apoptosis and cell proliferation.

Antibodies and Western blotting

Cells were harvested, after washing in ice-cold saline, by scraping and aspiration. For Western blotting studies, they were lysed in 9 M urea, 50 mM Tris–HCl (pH 7.3), and 0.15 M β-mercaptoethanol, and proteins were fractionated by SDS-PAGE in the presence 0.1 M Tris, 0.1 M Bicine, and 0.1% of SDS. After electrophoretic transfer to nitrocellulose membranes, antigens were detected using appropriate antibodies. Ad5AdE1A was detected with the mouse monoclonals M58 (Pharmingen) and M73, whereas p53 was detected with the monoclonal antibody DO1. β-actin was detected with a mouse monoclonal antibody (clone ac-74, Sigma). Ad5 structural proteins were detected with a rabbit polyclonal antibody (R1/99) raised against inactivated virus.

Apoptosis assays

Floating and adherent cells were pooled and apoptotic cells were identified and counted by staining the cells with acridine orange dye and examining for apoptotic characteristics under fluorescent microscopy. Cells with condensed and fragmented nuclei were counted as apoptotic. For apoptosis analysis in the proteasome inhibitor experiment, trypan blue exclusion assays were performed according to the instruction by the manufacturer (Sigma). At least 200 cells were counted in each experiment. In addition, fluorescence activated cell sorter (FACS) analysis was

carried out to detect propidium iodide-labeled apoptotic cells in a sub-G1 population, as described previously (Zhang et al., 2004).

Cell proliferation analysis

AlamarBlue™ reduction assay (Serotec Inc.) has been used for evaluation of cell proliferation. Cells were cultured in 96-well plates and infected with Ad5 viruses for 24 h before incubation with TNFα and/or lactacystin for a further 24 h. Then, alamarBlue dye was added to the medium and the cells were incubated for 4 h at 37 °C for color development: pink indicated growing viable cells while the blue suggested cell death according to the manufacturer's instruction. The relative cell growth rate was determined by analyzing optimal density data acquired from a multi-well plate fluorometer at an excitation wavelength of 530 nm and emission wavelength of 590 nm. The fluorescence of the samples was corrected by comparison with background fluorescence as determined by the blank sample on each plate using software Ascent.

Statistics

Student's *t* test was used to compare data between groups while ANOVA test was applied to compare the data among different groups. $P < 0.05$ was considered to be statistically significant. Mean ± SD was used to indicate the average and standard deviation values from at least two independent experiments with duplicate or triplicate samples.

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