Biochemical and Biophysical Research Communications **269,** 179–190 (2000)
doi:10.1006/bbrc.2000.2247, available online at http://www.idealibrary.com on IDEAL®

p21$^{WAF1/CIP1}$ Inhibits Initiator Caspase Cleavage by TRAIL Death Receptor DR4

Shi-Qiong Xu and Wafik S. El-Deiry[1]

Laboratory of Molecular Oncology and Cell Cycle Regulation, Howard Hughes Medical Institute, Departments of Medicine, Genetics, Cancer Center and Institute for Human Gene Therapy, University of Pennsylvania School of Medicine, 437 CRB, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104

Received January 19, 2000

Death receptors of the Tumor Necrosis Factor (TNF) family form membrane-bound self-activating signaling complexes that initiate apoptosis through cleavage of proximal caspases including CASP8 and 10. Here we show that overexpression of the cytoplasmic domain (CD) of the DR4 TRAIL receptor (TNFRSF10A, TRAIL R1) in human breast, lung, and colon cancer cell lines, using an adenovirus vector (Ad-DR4-CD), leads to p53-independent apoptotic cell death involving cleavage of CASP8 and 10 proximally and CASP3, 6, and 7 distally. DR4-CD overexpression also leads to cleavage of poly(ADP-ribose) polymerase (PARP) and the DNA fragmentation factor (DFF45; ICAD). Importantly, normal lung fibroblasts are resistant to DR4-CD overexpression and show no evidence of PARP-, CASP8- or CASP3-cleavage despite similar levels of adenovirus-delivered DR4-CD protein as the cancer cells. These results suggest that DR4 may signal death through known caspases and that further studies are required to evaluate Ad-DR4-CD as a novel anti-cancer agent. Finally, we show that overexpression of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ (CDKN1A), or its N-terminal 91 amino acids containing cell cycle-inhibitory activity, inhibits DR4-CD-dependent proximal caspase cleavage. The blockage of initiator caspase activation provides a novel insight into how p21 may suppress apoptosis and enhance cell survival.

Key Words: **apoptosis; death receptor; DR4; TRAIL; caspase; p21$^{WAF1/CIP1}$; gene therapy; cancer; PARP; DFF45.**

Pro-apoptotic members of the Tumor Necrosis Factor (TNF) receptor family engage the apoptotic cascade through the formation of a membrane bound death-inducing signaling complex (DISC) that includes the cytotoxic ligand, trimerized death receptor, adaptor and proximal caspase (see 1, 2 for review). A characteristic cytoplasmic death domain sequence in death receptors serves to recruit adaptor molecules which recruit caspases using their death effector domain. Through autocatalytic cleavage a mature proximal caspase is generated which in turn diffuses into the cytoplasm and activates downstream "executioner" caspases that mediate cell death. Proximal or initiator caspases include CASP8 and 10 (3–9), whereas distal or executioner caspases include CASP3, 6 and 7 (4, 9, 10). In addition, CASP2 and 9 are also believed to be involved in death signaling after being released from the mitochondria and activated by interacting with CARD containing proteins (4, 11–15). DISC function is subject to direct regulation by activators such as FLASH in the case of the Fas receptor (16) or inhibitors such as the suppressor of death domains (SODD) in the case of TNFR1 (17) or Fas decoy in the case of Fas (18). The TNF-related apoptosis-inducing ligand (TRAIL) receptor family includes two proapoptotic members DR4 (TNFRSF10A, TRAIL R1) and KILLER/DR5 (19–25) and two decoy receptors TRID and TRUNDD (25–28).

The pro-apoptotic TRAIL receptors are potent inducers of apoptosis through an as yet unclear mechanism. For example, whereas there is some evidence for Fas-associated death domain (FADD) adaptor-dependent death signaling by death receptors (22, 23), FADD may not be required for DR4-induced cell death signaling (19, 21, 28, 29). More recently, FADD knockout cells were found to undergo TRAIL-mediated apoptosis, further supporting the notion that TRAIL receptor-mediated apoptosis can occur through a FADD-independent pathway (30). However, little is known about the involvement of specific initiator or execu-

[1] To whom correspondence should be addressed. Fax: (215) 573-9139. E-mail: weldeir@hhmi.upenn.edu.

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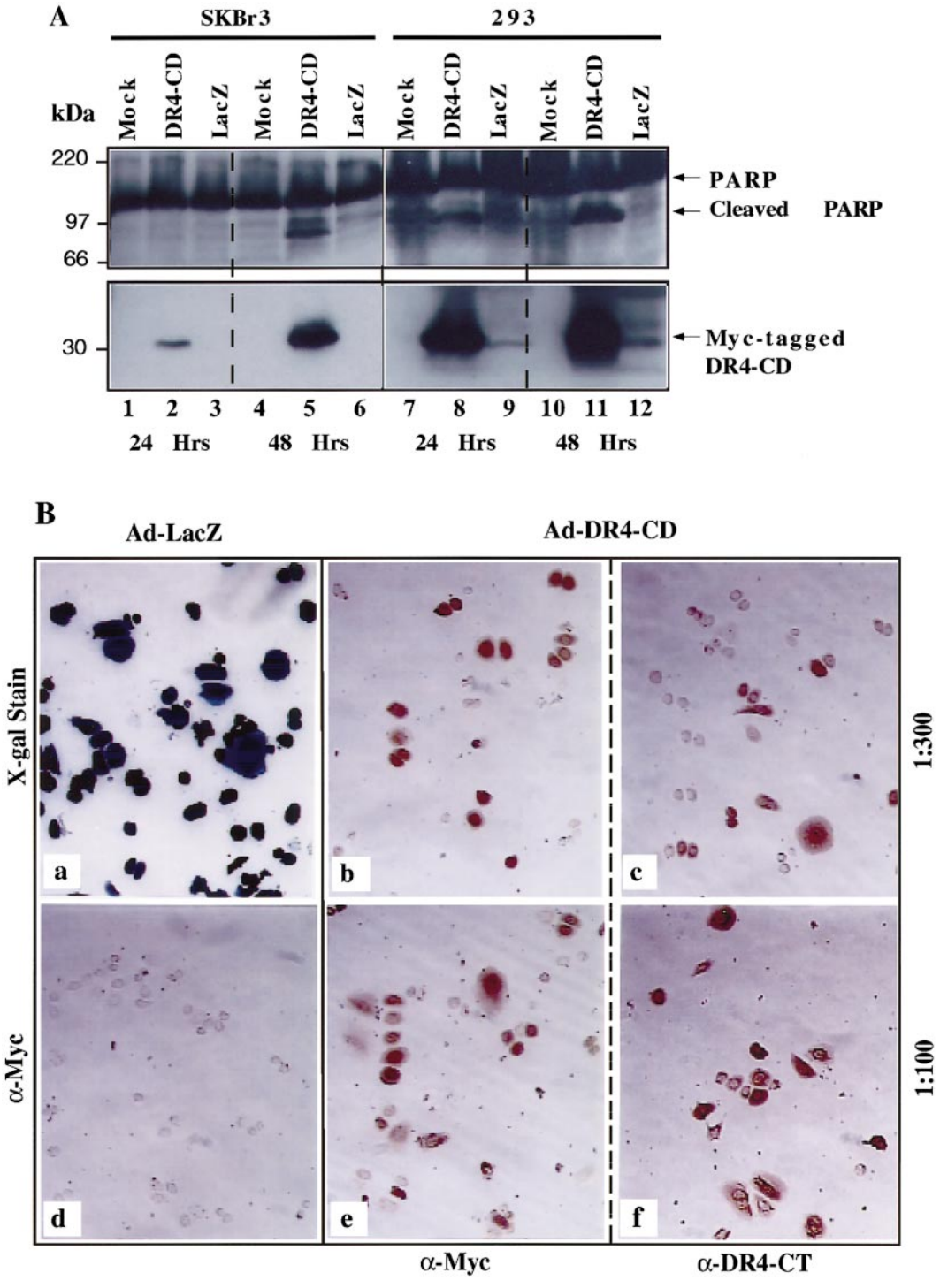
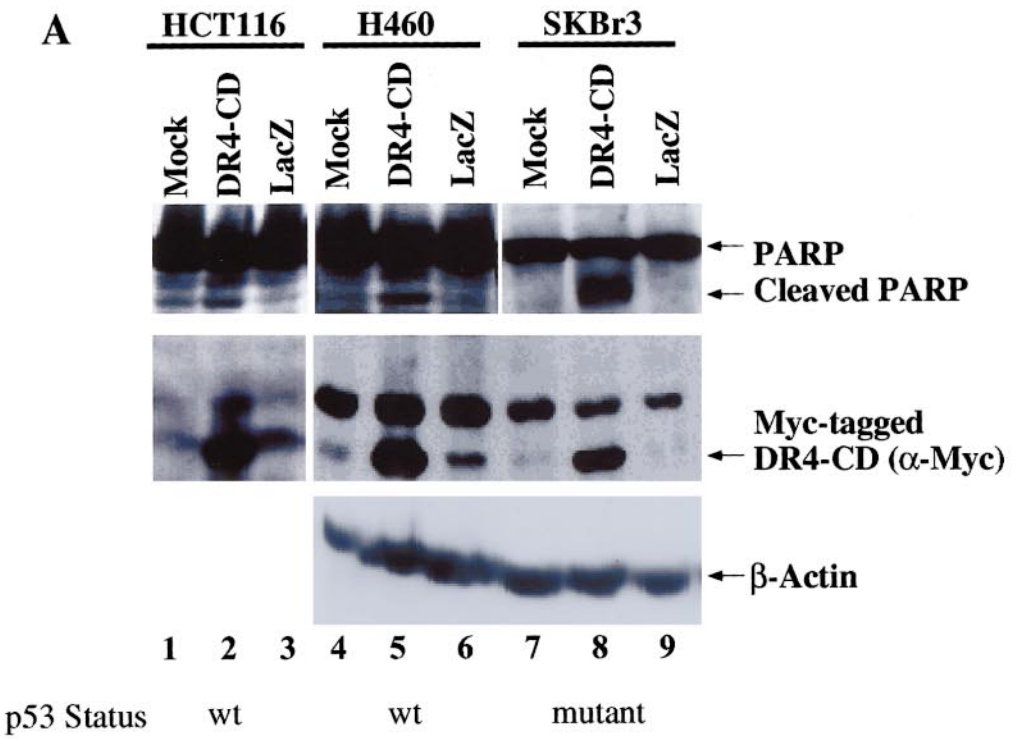


FIG. 1. DR4-CD protein expression and PARP cleavage following infection of 293 and SKBr3 cells by Ad-DR4-CD. (A) A total of 5×10^5 each of 293 or SKBr3 cells were infected or left uninfected for 24 and 48 h. Cell lysates were collected and 100 μg of proteins from each lysate were analyzed using 10% SDS–PAGE followed by immunoblotting with individual antibodies for PARP cleavage and myc-tagged DR4-CD expression. DR4-CD, Ad-DR4-CD; LacZ, Ad-LacZ. (B) A total of 5×10^3 of SKBr3 cells seeded overnight in 24-well plates were infected with the indicated viral preparation at two different dilutions (1:300 and 1:100) for 16 h. X-gal stain was applied to Ad-LacZ-infected cells to measure the infectivity (a). Immunocytochemistry was used to detect DR4-CD expression in Ad-LacZ-infected (d) or Ad-DR4-CD-infected (b, c, e, and f) cells with antibodies either against the myc epitope (b, d, and e) or against the C-terminus of DR4 (c, f).


A
HCT116
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SKBr3
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DR4-CD
LacZ
Mock
DR4-CD
LacZ
Mock
DR4-CD
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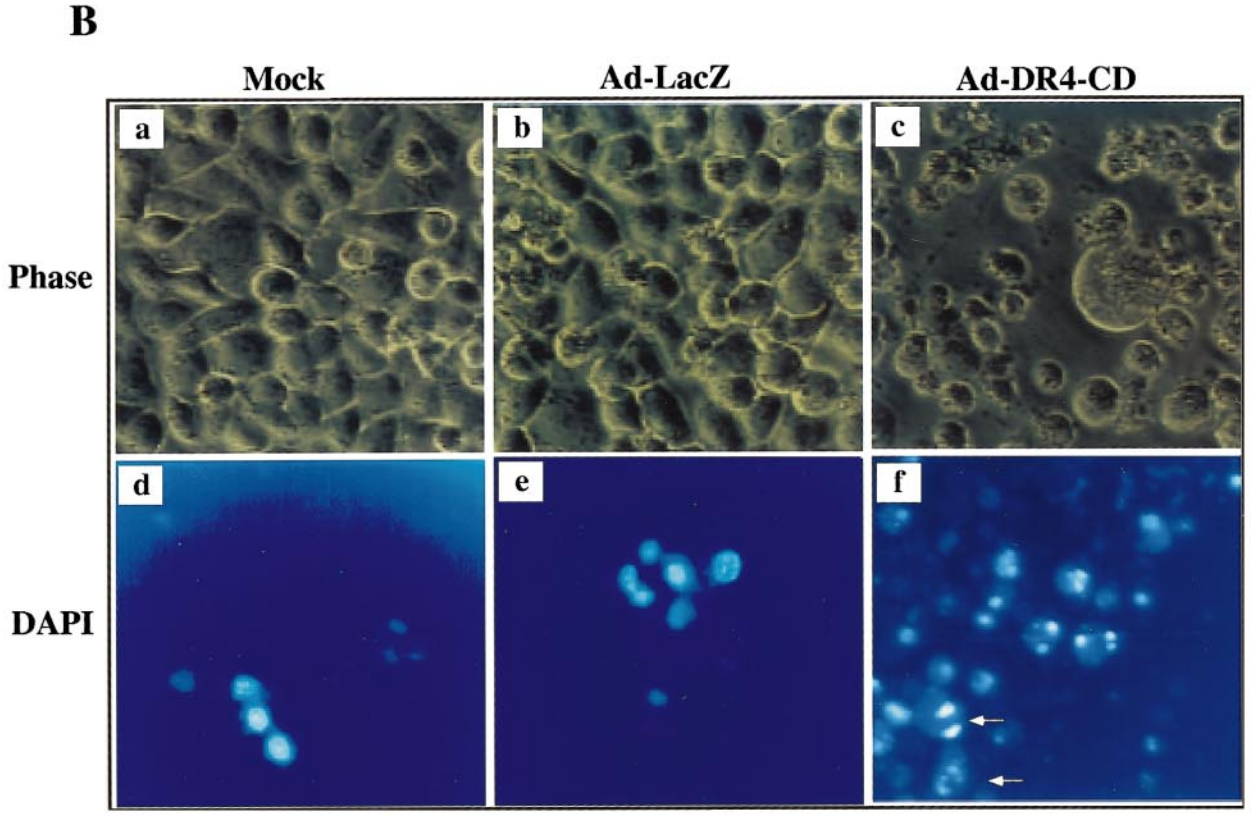
Myc-tagged
DR4-CD (α-Myc)
β-Actin
1 2 3 4 5 6 7 8 9
p53 Status wt wt mutant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tioner caspases downstream of TRAIL death receptors.

In the present studies we employed an adenovirus vector to express high levels of the cytoplasmic domain of DR4 in human cancer cell lines. The Ad-DR4-CD vector delivered the DR4-CD protein and induced apoptosis of colon, breast and lung cancer cell lines as evidenced by morphologic features, rapid DAPI staining, and PARP cleavage. We further explored the mechanism by which the cytoplasmic domain of DR4 engages the caspase cascade. We found evidence for cleavage of initiator and executioner caspases as well as DFF-35/45 ($ICAD_{S/L}$), a subunit of the heterodimeric DNA fragmentation factor (DFF) which inhibits caspase-activated DNase (DFF-40/CAD) (31–45). Ad-DR4-CD induced death of cancer cells regardless of p53 status, but normal lung and foreskin fibroblasts were resistant to apoptosis despite similar levels of DR4 protein expression as the cancer cells. These results suggest that Ad-DR4-CD may have selectivity for killing of cancer cells versus normal cells.

The cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (46) inhibits cell cycle progression in response to DNA damage. p21 also appears to protect some cells against apoptosis induced by variety of cytotoxic agents. For instance, mimosine-induced p21 expression has been shown to prevent apoptosis induced by UV-irradiation or treatment with an RNA polymerase II inhibitor (47). We recently reported that ectopic $p21^{WAF1/CIP1}$ can protect cells against the cytotoxic effects of etoposide possibly through cell cycle arrest and prevention of DNA damage (48). These observations indicate a p21-dependent G1 checkpoint of apoptosis. However, other recent studies have suggested that $p21^{WAF1/CIP1}$ may more directly influence apoptotic signaling events, perhaps independently of its cell cycle inhibitory effect (49–51). We investigated the effect of p21 on DR4-induced apoptosis and found that p21 could inhibit the DR4 activated death signal at the level of proximal caspase cleavage. This novel activity of p21 was mapped to its amino-terminus and suggests a model by which p21 may inhibit apoptosis through an early step prior to initiation of the caspase cascade.

MATERIALS AND METHODS

Cell lines, tissue culture, and transfection conditions. Six cell lines were used in this study. HCT116, obtained from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD), is a human colon carcinoma cell line with wild-type p53; H460, obtained from Dr. S. B. Baylin (Johns Hopkins University) is a human non-small cell lung cancer cell line with wild-type p53; SKBr3 is a human breast adenocarcinoma cell line with mutant p53; WI38 is a normal human lung diploid fibroblast cell line with wild-type p53; HS27 is a normal human foreskin fibroblast cell line; and 293 is a kidney cell line. SKBr3, WI38, HS27 and 293 cell lines were bought from ATCC and maintained routinely in Dulbecco's modified Eagle's medium (DMEM), while HCT116 and H460 were maintained as described previously (52). All the media contain 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. Transfections were carried out as previously described (53).

Cloning of the cytoplasmic domain of human DR4. The cytoplasmic domain of human DR4 were isolated by reverse transcription-PCR from mRNA extracted from H460 cells, using a combination of TA cloning and subcloning into the expression vector pcDNA 3.1A. Primers used for initial amplification were: forward primer, 5′-CCGGAATTC*CCACC*ATGGGAGGGGACCCCAAGTGC; and reverse primer, 5′-CCCAAGCTTCTCCAAGGACACGGCAGA. RT-PCR was carried out as described previously (54). The PCR conditions were: amplification for 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 4 min. The amplified fragment containing EcoRI and HindIII sites was purified following QIAquick Gel Extraction Kit Protocol (QIAGEN) and then cloned into pCRII vector using the One Step TA cloning kit (Invitrogen), to generate the pCRII-TOPO-DR4-CD vector. The DNA sequence of the cytoplasmic domain of human DR4 was verified. The DR4-CD insert was released as a EcoRI/HindIII fragment and subcloned into EcoRI-HindIII sites of the pcDNA 3.1A vector (Invitrogen). The orientation and sequence of the cloned DR4-CD insert were verified. The DR4-CD insert was PCR amplified in frame with the myc tag using the following primers: the forward primer 5′-CGCGGATCCGAATTCCCACCATGGGAGGGGACC, and the reverse primer 5′-CGCGGATCCTCAGTCGACGGCGCTATTCAGATC. The amplified BamHI-fragment was purified following the same protocol as for the EcoRI-HindIII fragment and then subcloned into the BamHI site of the expression vector pMV60-Bam (55). This procedure generated two constructs expressing DR4-CD-Myc, designated pMV60-DR4-CD-Myc, clone 15 and 17. The DNA sequence and expression (*in vitro* translation and Western blotting after transfection of human cells) of this DR4-CD-Myc was confirmed (data not shown).

Adenovirus infections. Ad-p21, Ad-p21N, Ad-cMyc and Ad-LacZ were previously described (54–57). The pMV60-DR4-CD-Myc clone 15 was cotransfected with the pJM17 adenovirus backbone vector into 293 cells following a procedure described previously (54–57),

FIG. 2. Ad-DR4-CD-induced apoptosis in various human cancer cell lines is independent of p53 status. Ad-DR4-CD infection of HCT116 (human colon carcinoma; wt p53), H460 (human non-small cell lung cancer; wt p53), and SKBr3 (human breast adenocarcinoma; mutant p53) led to massive apoptosis as assessed by detection of PARP cleavage, morphological change, and nuclear fragmentation. (A) A total of 5×10^5 cells seeded overnight in 6-well plates were infected with adenoviruses expressing either human DR4-CD or LacZ or left uninfected (Mock) for 24 h and harvested for Western analysis. A total of 100 μg of protein from each lysate was separated by 10% SDS–PAGE followed by immunoblotting using various antibodies as indicated. β-Actin was used to confirm equivalent protein loading. DR4-CD, Ad-DR4-CD; LacZ, Ad-LacZ. (B) Morphological change, chromatin condensation and fragmentation in nuclei of Ad-DR4-CD-infected SKBr3 cells. SKBr3 cells treated as in A were also observed by phase contrast microscopy and DAPI staining. The Ad-DR4-CD-infected cells (c) displayed cell shrinkage, surface blebbing, rounding up and detaching from culture surface, compared with the uninfected (a) or Ad-LacZ-infected cells (b). The nuclei of cells sampled from a–c were stained with 4′6-diamidino-2-phenylindole (DAPI) and observed under a UV microscope. Arrows show condensed chromatin and fragmented nuclei in Ad-DR4-CD-infected SKBr3 cells (f) that are not found in the uninfected (d) or Ad-LacZ-infected cells (e).

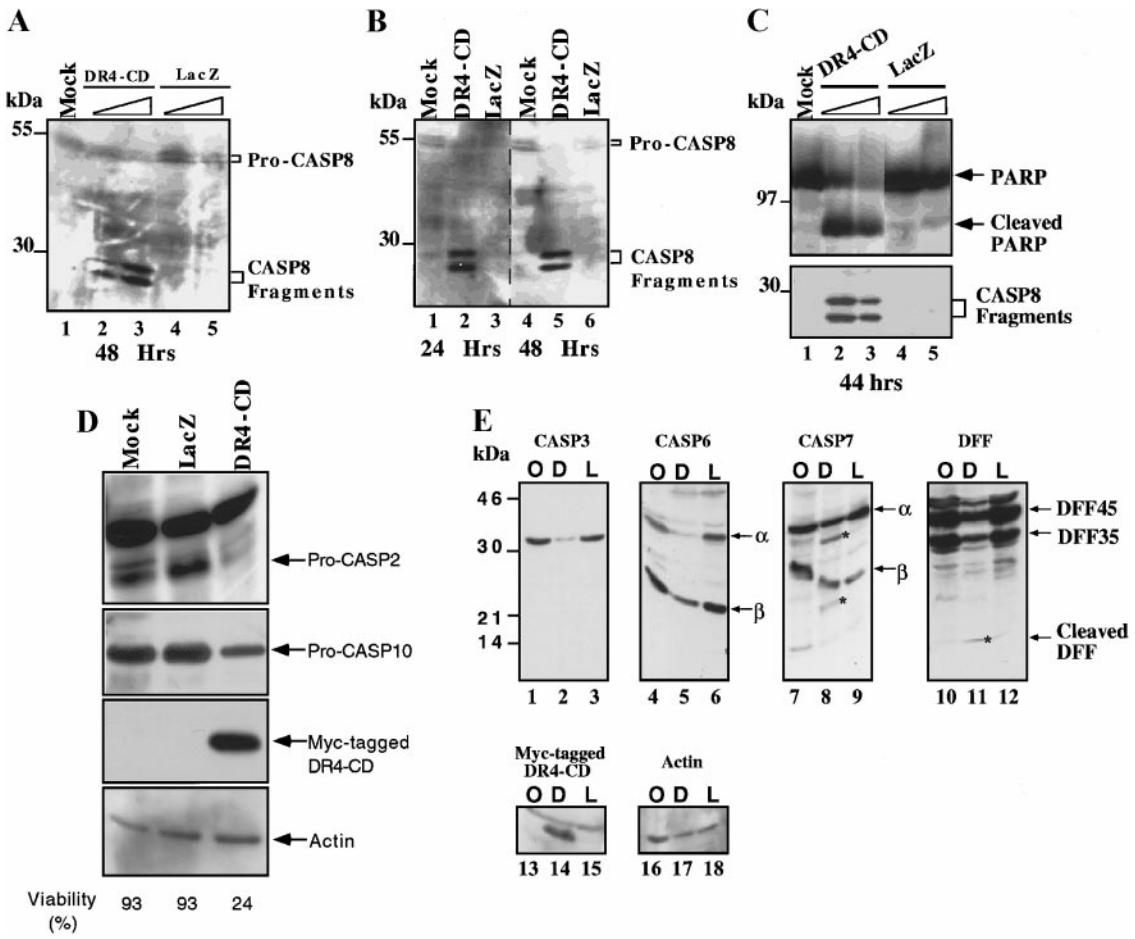
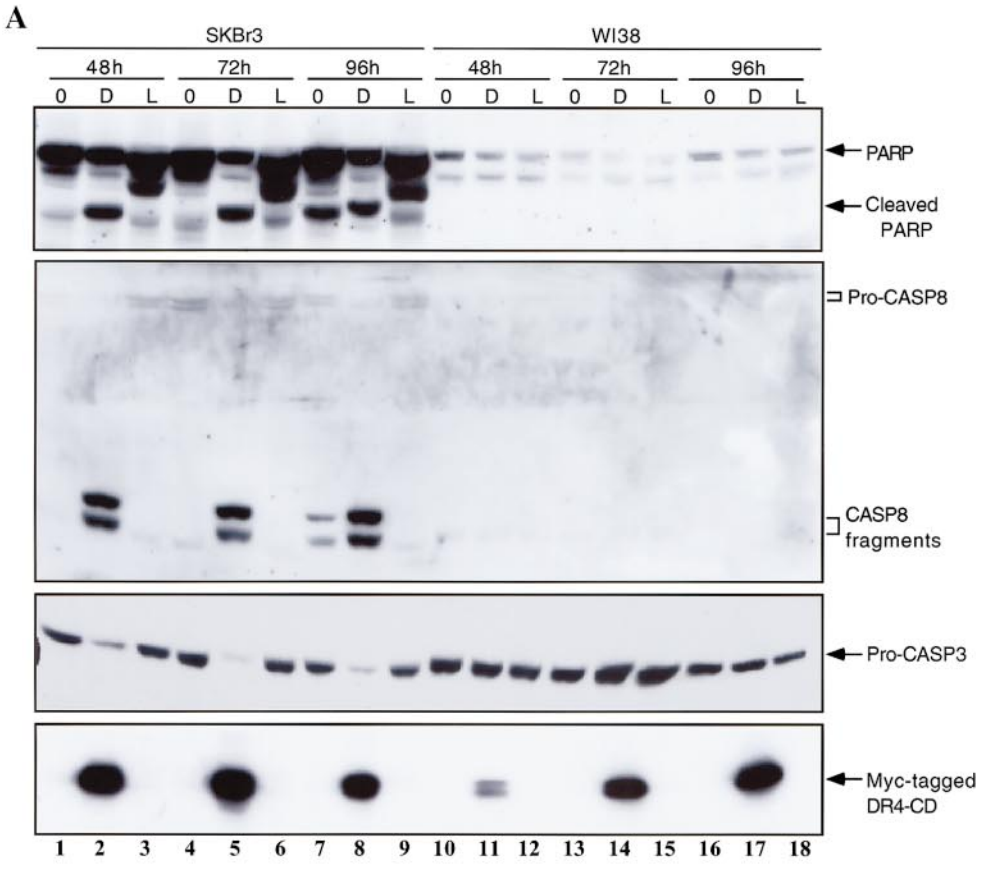


FIG. 3. Ad-DR4-CD-induced apoptosis involves activation of CASP2, 8, 10 proximally and 3, 6, 7 distally as well as DFF. (A–C) Ad-DR4-CD-induced caspase-8 activation correlates with PARP cleavage. SKBr3 cells were infected with Ad-DR4-CD or Ad-LacZ or left uninfected for the different time points (A, 48 h; B, 24 and 48 h; and C, 44 h). A total of 60 μg of protein from the cell lysate of each treatment was analyzed by 15% SDS–PAGE (A, B and lower C) or 10% SDS–PAGE (upper C) followed by immunoblotting using antibodies against either caspase-8 (A, B, and lower C) or PARP (upper C). The concentration gradient triangle represents increasing adenovirus used from 25 to 50 PFUs/Cell in A and from 50 to 250 PFUs/Cell in C, respectively. (D) Ad-DR4-CD-induced cleavage of caspase-2 and 10 and decreased cell viability. SKBr3 cells were infected with Ad-DR4-CD or Ad-LacZ or left uninfected for 48 h. A total of 60 μg of protein from the cell lysate of each treatment was separated by 10% SDS–PAGE (for caspase-10 blot) or 15% SDS–PAGE (for the remaining blots) followed by immunoblotting using antibodies against the indicated molecules. % viability was determined by trypan blue exclusion assay. (E) Cleavage of CASP 3, 6, 7 and DFF induced by DR4-CD. Western blotting was carried out as in D using 15% SDS–PAGE with the exception for CASP6, where 120 μg of protein was analyzed. O, Mock; D or DR4-CD, Ad-DR4-CD; L or LacZ, Ad-LacZ; *, cleaved product; α and β, two isoforms of the indicated caspases.

resulting in the recombinant virus Ad-DR4-CD. Adenovirus purification and infection was performed as previously described (54–57). The concentration of the recombinant adenovirus was measured based on the absorbance at 260 nm following purification on a cesium chloride gradient. Immunocytochemical methods were used to determine Ad-DR4-CD infectivity of normal and cancer cell lines (see Fig. 1B and 4B). Adenoviruses were used at a concentration that resulted in >90% infectivity of cell lines.

Western blot, immunocytochemistry, and X-gal stain. All Western blots were carried out as described previously (54–57). Floating dead cells were combined with scraped adherent cells for Western analysis. The primary antibodies used were: mouse anti-human monoclonal antibodies against Myc tag (c-Myc 9E10, Santa Cruz, 1:500 dilution), p21 (Oncogene Research, 1:500 dilution), caspase-3 (E8, Santa Cruz, 1:500), caspase-6 and -7 (Pharmingen, 1:500); rabbit anti-human polyclonal antibodies against C-terminal eitope of DR4 (α-DR4-CT, Pharmagen, 1:1000 dilution), PARP (Boehringer Mannheim, 1:2000 dilution), N-terminus of DFF (IMGENEX, #IMG-114, 1:500) and Caspase-2 (Santa Cruz, 1:200); goat anti-human polyclonal antibodies against caspase-8, 10 and β-actin (Santa Cruz, 1:200 dilution for caspases and 1:500 dilution for β-actin). Immunoblot analysis was performed with the horse-radish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit or mouse anti-goat IgG (PIERCE, 1:5000 dilution) based on their respective primary antibodies using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Life Science). Immunocytochemistry by HRP was performed in 24 well plates. After viral infection (5,000 cells/well for 16 h in Fig. 1B or 1×10^5 cells/well for 48 h in Fig. 4B), cells were subjected to immunocytochemical assay followed by photomicrography as described previously (54). X-gal stain was performed as described (56).


A
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48h
72h
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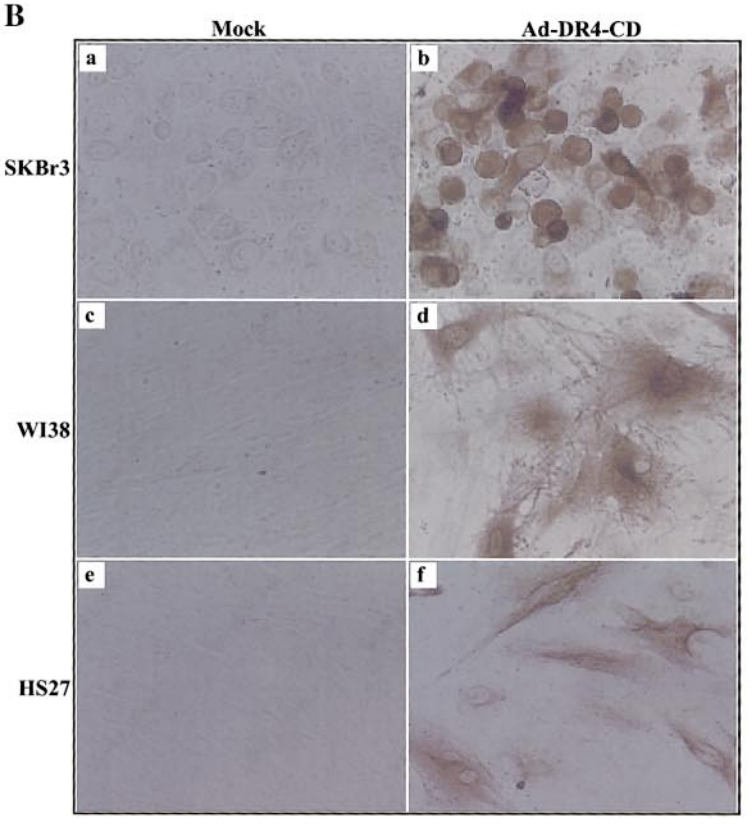
B
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RESULTS

Induction of cancer cell apoptosis by the cytoplasmic domain of DR4 is independent of p53 status. To investigate the cytoplasmic events in apoptotic signaling by DR4 we cloned the cytoplasmic domain of human DR4 in frame with a C-terminal myc tag into a replication deficient Ad5 adenovirus vector. Following infection of SkBr3 breast cancer or 293 kidney cells, expression of the myc-tagged DR4-CD protein was detected at 24 and 48 h post-infection (Fig. 1A). Cleavage of poly-ADP ribose polymerase (PARP) was observed in both cell lines by 48 h post-infection. Using immunocytochemical methods the expression of DR4 protein was confirmed in Ad-DR4-CD-infected SkBr3 cells using either anti-c-myc or anti-DR4 antibodies (Fig. 1B). PARP cleavage was observed following Ad-DR4-CD infection of mutant (SkBr3) or wild-type (HCT116, H460) p53-expressing cancer cells by 24 h (Fig. 2A). In addition to PARP cleavage, DR4-CD induced morphological changes consistent with apoptosis as observed under phase microscopy or UV-microscopy of DAPI-stained SKBr3 cells (Fig. 2B). Phase-contrast microscopic analysis showed that the Ad-DR4-CD infected cells had a decreased ability to spread on the tissue culture substrate. Many cells were rounded, detached from the culture surface and floated in the culture medium, displaying cell shrinkage and surface blebbing (c). In contrast, the Ad-LacZ infected cells (b) remained attached and grew as well as uninfected cells (a). To examine the effect of Ad-DR4-CD infection on chromatin integrity, cells were sampled from a–c and their nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under a UV-microscope. Condensed chromatin and fragmented nuclei were observed in Ad-DR4-CD infected SKBr3 cells (f) but not in uninfected (d) or Ad-LacZ infected cells (e). These studies demonstrate that the cytoplasmic domain of DR4 is sufficient to induce apoptosis of human cancer cells regardless of p53 status. Because only the cytoplasmic domain of DR4 was used, the observed apoptotic death is believed to be TRAIL ligand-independent.

Involvement of CASP8 and 10 proximally, CASP3, 6, and 7 distally, and DFF in DR4-induced death signaling. Of the three cancer cell lines examined, Ad-DR4-CD induced apoptosis most efficiently in the SkBr3 breast cancer cells. Thus we used this cell line for subsequent studies focussing on the identification of caspase activation downstream of the DR4 death signal. Although there is some data implicating physical association between DR4 and caspases 8 and 10 (21, 26), there has been no report of caspase cleavage and no information on the involvement of any downstream caspases. The results shown in Fig. 3 reveal that following infection of SkBr3 cells by Ad-DR4-CD there is cleavage of CASP8 and 10 proximally and CASP3, 6, and 7 distally in the apoptotic cascade. In addition, Cleavage of CASP2, a mitochondrial caspase (15) for which no downstream substrate has been identified, was induced by DR4-CD (Fig. 3D). In some cases, caspase cleavage was inferred from the depletion of procaspase protein from DR4-overexpressing but not control cells. For example, more than ninety percent of Pro-CASP-2, 3, and 6α and about two-thirds of Pro-CASP10 and 6β proteins were depleted from the DR4-overexpressing SKBr3 cells compared to the control cells. In other cases, caspase cleavage was directly observed by the detection of cleaved fragments, for example CASP7 and 8 (Fig. 3A–C, E). The activation of the caspase cascade by DR4-CD was well correlated with three other apoptotic markers: cleavage of PARP (Fig. 3C) and DFF (Fig. 3E) as well as a substantial decrease in cell viability as measured by trypan blue exclusion (Fig. 3D; % viability shown below lowermost panel).

Normal lung fibroblasts are resistant to Ad-DR4-CD-induced apoptosis. Because replication-deficient adenovirus can be utilized as a gene delivery vector, we investigated the effects of Ad-DR4-CD on normal human cells to explore a potential application of this recombinant adenovirus in cancer gene therapy. Figure 4A shows that WI38 normal diploid lung fibroblasts are resistant to DR4-CD induced apoptosis. A similar level of DR4-CD expression induced cleavage of PARP, CASP8 and CASP3 in SKBr3 breast cancer cells but not in the WI38 cells (Fig. 4A, lanes 2, 5, 8 vs lanes 11, 14, 17). Figure 4B shows that, at 48 h post-infection, many DR4-CD overexpressing SKBr3 cells were rounded, and detached from the culture surface (b) as compared to the uninfected cells (a). In contrast, Ad-DR4-CD infected WI38 cells appeared healthy, attached (d) and grew as well as uninfected cells (c).

FIG. 4. Normal human WI38 lung fibroblasts are resistant to Ad-DR4-CD-induced apoptosis. (A) A total of 1×10^5 cells each of SKBr3 and WI38 were seeded in 24-well plates overnight, and then infected with Ad-LacZ or Ad-DR4-CD or left uninfected for 48, 72 and 96 h. A total of 20 μg of protein from the lysate of each treatment was subjected to Western blot following 10% SDS–PAGE to analyze for PARP cleavage and DR4-CD expression, and 15% SDS–PAGE for CASP3, 8 cleavage. A similar level of DR4-CD expression induced cleavage of PARP, CASP8, 3 in the SKBr3 cells but not in the WI38 cells. O, mock; D, Ad-DR4-CD; L, Ad-LacZ. (B) A total of 1×10^5 of cancer (SKBr3) or normal (WI38 and HS27) cells seeded overnight in 24-well plates were infected with Ad-DR4-CD or left uninfected (mock) for 48 h followed by immunocytochemistry to detect DR4-CD expression in uninfected (a, c, and e) or Ad-DR4-CD-infected (b, d, and f) cells with antibody against the C-terminus of DR4.

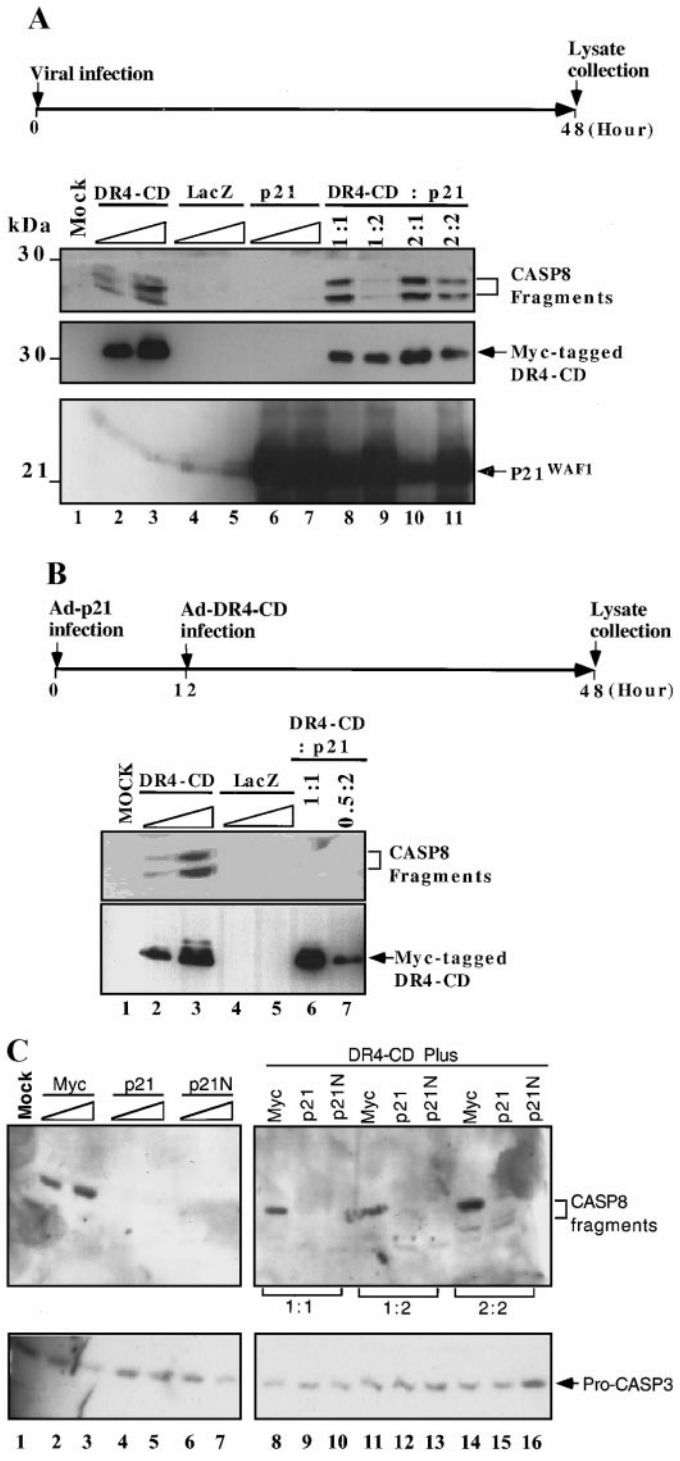

FIG. 5. Overexpression of $p21^{WAF1}$ blocked Ad-DR4-CD-induced CASP8 cleavage. (A) A total of 5×10^5 of SKBr3 cells were seeded in 6-well plates overnight and then infected with adenoviruses expressing DR4-CD, LacZ or $p21^{WAF1}$ or with different combinations of DR4-CD and $p21^{WAF1}$ (as indicated), or left uninfected (mock). At 48 h after infection, cell lysates were collected and 60 μg of protein from each treatment was analyzed for DR4-CD and $p21^{WAF1}$ expression as well as CASP8 cleavage (see Materials and Methods). (B) SKBr3 cells were allowed to express the $p21^{WAF1}$ protein by Ad-p21 infection first for 12 h, then Ad-DR4-CD at two indicated ratios (lanes 6, 7) for 36 h. Initially, uninfected cells were also infected at 12 h with Ad-DR4-CD (lanes 2, 3) or Ad-LacZ (lanes 4, 5) or left uninfected (mock). A total of 60 μg of protein lysate from each treatment was examined for CASP8 fragments and DR4-CD expression as in A. (C) The antiapoptotic activity of p21 was mapped to its N-terminus. The infection procedure described in B was used except for using c-Myc delivering adenovirus as a control, including adenovirus delivering the N-terminal 91 amino acids of p21 containing cell cycle-inhibitory activity. The same blot was stripped and reprobed for Pro-CASP3 as a protein loading control. The concentration gradient triangle represents increasing adenovirus used from 50 to 100 PFUs/Cell. At a ratio of 1:1 each adenovirus was used at approximately 100 PFUs/Cell. DR4-CD, Ad-DR4-CD; p21, Ad-p21; p21N, adenovirus delivering the N-terminal 91 amino acids of p21; Myc, Ad-c-Myc; LacZ, Ad-LacZ.

These differences lasted for up to 96 h (Fig. 4A and data not shown). We extended this observation to another normal cell line, human foreskin fibroblasts HS27. Although HS27 cells required a higher viral dosage to reach a similar level of infectivity, we found that this normal cell line is also resistant to DR4-CD induced apoptosis (Fig. 4B-e, f and data not shown). Our studies provide evidence that Ad-DR4-CD preferentially kills cancer cells versus normal cells and thus further studies may be warranted to explore its use in gene therapy of cancer.

$p21^{WAF1/CIP1}$ inhibits Ad-DR4-CD-induced apoptosis and proximal caspase cleavage. In the present studies we sought to further clarify the mechanism by which p21 may affect death signaling by DR4-CD. We found that overexpression of $p21^{WAF1}$ can inhibit DR4-CD-induced CASP8 cleavage (Fig. 5). The blocking effect depended on the ratio between DR4-CD and p21 (Fig. 5A, lanes 8–11). When cells were allowed to overexpress $p21^{WAF1}$ protein before the Ad-DR4-CD infection, overexpression of $p21^{WAF1}$ completely blocked the DR4-CD induced cleavage of CASP8 (Fig. 5B). Furthermore, when compared with a control adenovirus expressing c-Myc (Fig. 5C, lanes 8, 11, 14), Adenovirus delivered p21 N-terminal 91 amino acids containing cell cycle-inhibitory activity blocked DR4-CD-dependent proximal CASP8 cleavage (Fig. 5C, lanes 10, 13, 16) as efficiently as a full length of p21 protein (Fig. 5C, lanes 9, 12, 15), mapping this novel activity of p21 to its amino-terminus. These data suggest that $p21^{WAF1}$ protects cells from apoptosis possibly through an early step prior to initiation of the caspase cascade and this novel activity resides within its N-terminus.

DISCUSSION

The cytoplasmic domain of the DR4 TRAIL receptor (TNFRSF10A, TRAIL R1) was demonstrated to induce apoptosis, independent of p53 status, in human lung,

colon and breast cancer cell lines (Figs. 1 and 2), but not in normal human lung or foreskin fibroblasts (Fig. 4). The cytoplasmic domain of human DR4 was selected because we assumed that any part of this domain may contribute to signaling death, although the death domain region shared by the TRAIL receptor family is believed to possess the critical pro-apoptotic signal. It is generally believed that the death receptors become activated upon binding of their cognate ligands in a process that involves trimerization in the membrane, recruitment of adaptors and initiator caspases, and formation of a complex called death-inducing signaling complex (DISC) *in vivo* (see the introduction). Because only the cytoplasmic domain of DR4 was used in this study, the observed apoptotic death is believed to be TRAIL ligand-independent. Clearly the requirement for the extracellular ligand binding domain and the transmembrane domain is not an absolute one, because a cytoplasmic domain can cause cell death. Moreover, the specificity of ligand binding and competition by decoy receptors can be circumvented or bypassed through the expression of the cytoplasmic domain of a pro-apoptotic death receptor. This is not surprising because the death domain is believed to be critical for the formation of the DISC and the activation of downstream events (1, 2, 6, 7, 58). Additional studies are required to demonstrate any potential therapeutic value of this reagent using additional cancer and normal cell lines in cell culture and animal models. It was somewhat surprising that normal cells are resistant to ectopic expression of the DR4-CD given the currently held belief that decoy receptors confer TRAIL resistance.

The cell death pathway activated by the DR4-CD seems to involve both CASP8 and 10 (Figs. 3A–3D). However, how these initiator caspases become activated is yet to be defined. A large molecule called FLASH that is homologous to CED-4 has recently been shown to be capable of interacting with and activating CASP8 (16). Most recently, c-E10, a DR4 interacting protein homologous to v-E10, has been identified but shown to possess little apoptotic activity (59). Whether the FLASH or c-E10 take part in the DR4-CD's DISC-like complex remains to be clarified.

It was revealed in this study that all three known executioner caspases, CASP3, 6 and 7, become activated in the apoptotic signaling pathway of DR4 in SKBr3 cells (Fig. 3E). This statement is supported by several lines of evidence: (a) CASP3 was cleaved following overexpression of DR4-CD (Figs. 3E and 4A). (b) Both isoforms of CASP6 were cleaved (Fig. 3E). (c) CASP7α was partially cleaved into intermediate or subunit products (Fig. 3E) (9, 60). (d) MCF-7, a cell line deficient in caspase-3 function (42, 61) has been shown to undergo apoptosis after treatment with TRAIL or ectopic expression of DR4 (19, 21), suggesting that either CASP6 or CASP7 or both or another unidentified CASP3-like caspase may mediate the DR4 death signal. (e) Both CASP8 and 10 have been shown to activate CASP 3, 6 and 7 (3, 4, 8–10). (f) The activation of the caspase cascade was correlated with cleavage of PARP, DFF as well as substantial cell death (Fig. 1A, 2A, 3C–E, and 4A). Both CASP3 and 7 have been demonstrated to cleave DFF45/ICAD (62 and below). We therefore conclude that CASP3, 6 and 7 may be involved in DR4 apoptotic signaling at the executioner level, although it is possible that the ultimate activation of all downstream caspases may not be the result of a direct linear pathway.

In the present study, both DFF35 ($ICAD_S$) and DFF45 ($ICAD_L$) were cleaved to release the DNase subunit DFF40 (CAD) (36, 37, 63). As a substrate, DFF35 ($ICAD_S$) and 45 ($ICAD_L$) can be cleaved by CASP3 and 7 but not by CASP6 and 8 (62). Cleaved ICAD dissociates from DFF40 (CAD), allowing the latter to oligomerize, forming a large functional complex that degrades DNA by introducing double strand breaks (62). In this context, the chromatin-associated protein histone H1 and high mobility group proteins directly interact with DFF, confer DNA binding ability to DFF, and markedly stimulate the intrinsic DNase activity of DFF40 (CAD) (39, 62). Our result is consistent with several recent reports that inhibitory DFF subunits are cleaved by treatment with diverse apoptotic stimuli such as anti-Fas antibody (44), TNFα (42), staurosporine (34, 36, 42), etoposide, UV- or γ-radiation (63).

The observation that CASP2 cleavage occurs in response to DR4-CD is an indication that the barrier function of mitochondrial membranes is perturbed early during DR4-induced cell death signaling. CASP2 plays a central role in mediating both normal and pathophysiological apoptosis in female germ cell lineage (13). CASP2 zymogen is essentially localized in mitochondria, redistributed to the cytosol and processed to generate enzymatically active caspase due to the disruption of the outer mitochondrial membrane occurring early during apoptosis (15). It is assumed that the DR4-induced release of mitochondrial caspases may be mediated by pro-apoptotic Bcl2 family members such as Bid, through the action of caspase 8 (64–66). However, it remains to be investigated whether Bid is cleaved downstream of DR4.

It is believed that $p21^{WAF1}$ can protect cells from apoptosis but the underlying mechanism is yet to be defined (46). There are several well documented reports of protection by p21 from apoptosis. p21 was shown to inhibit apoptosis following prostaglandin exposure or during muscle differentiation (67, 68). We recently reported that ectopic p21 can protect cells against the cytotoxic effects of etoposide (48). Mimosine-induced p21 expression has been shown to

prevent apoptosis induced by UV-irradiation or treatment with an RNA polymerase II inhibitor (47). $p21^{WAF1/CIP1}$ −/− cells have been found to be more sensitive to the cytotoxic effects of ultraviolet or ionizing radiation and other DNA damaging agents such as cisplatin (69, 70). The mechanism of protection by $p21^{WAF1/CIP1}$ is believed to involve cell cycle arrest that may prevent damage or which may permit repair of existing DNA damage (48, 71). Failure to arrest allows replication of a damaged DNA template with subsequent lethality. Agents such as etopside can cause lethal double strand breaks as a replication fork proceeds through a topoisomerase II-etoposide inhibited DNA complex. However, it is possible that p21 may inhibit apoptosis through more direct mechanisms that may be independent of its role as a cell cycle inhibitor (47).

Our present studies indicate that overexpression of $p21^{WAF1}$ can block DR4-CD-induced CASP8 cleavage (Fig. 5). This effect is more prominent if a time window is given to produce the p21 protein before initiation of the DR4-CD death signal. These data suggest that p21 may protect cells from apoptosis possibly through an early step prior to amplification of the caspase cascade. One of the unanswered questions raised by this work involves the mechanism of protection from apoptosis through inhibition of CASP8 cleavage by p21. Our previous studies showed that p21 can protect cells from etoposide induced apoptosis at least in part through prevention of DNA damage due to cell cycle arrest (48). In the case of etoposide, a "cell cycle-active" agent, toxicity occurs following DNA damage during S-phase. It is therefore expected that a p21-mediated G1 arrest may prevent apoptosis by virtue of the protective effect of the cell cycle arrest on damage accumulation. To our knowledge there has been no suggestion that death receptors or cytotoxic ligands are cell cycle-specific in their mode of killing. Similarly there is no data to suggest that initiator caspases are more or less likely to be activated or cleaved in G1 phase versus other phases of the cell cycle, or in arrested versus proliferating cells. Thus in contrast to the case of cell-cycle active agents, we suspect that p21 may have a protective effect against death receptor-induced cell death, above and beyond its CDK-inhibitory activity. In this regard there is precedent for p21 inhibition of cytoplasmic kinases. Cytoplasmic $p21^{WAF1}$ can form a complex with ASK1 resulting in inhibition of ASK1 and SAPK/JNK (49). Phosphorylation of caspase-9 by akt has also been shown to confer resistance to apoptosis (72). Therefore, we speculate that p21 mediated effects on kinases other than CDKs may underlie its apoptosis-inhibitory effect. However, the precise mechanism of protection against cell death by p21 remains to be determined. Our data shows that there is no difference in the expression levels of the ectopically-expressed cytoplasmic domain of DR4 or of initiator caspases (Fig. 5 and data not shown). It is possible, through direct or indirect effects, that p21 inhibits initiator caspase cleavage leading to enhanced cell survival. This is a critical point in cell signaling that precedes amplification of the caspase cascade and could represent a potentially effective mechanism through which p21 may prevent apoptosis.

In conclusion, we generated an adenovirus expressing the cytoplasmic domain of human DR4 (Ad-DR4-CD). The DR4 signaling pathway has been demonstrated to involve CASP8 and 10 proximally and CASP 3, 6 and 7 distally, finally leading to cleavage of PARP and DFF. The Ad-DR4-CD reagent should be further studied for a possible use in cancer gene therapy because unlike cancer cells, normal lung or skin fibroblasts were resistant to its killing effects. Finally our studies provide evidence for a novel activity of p21 to inhibit initiator caspase cleavage as an early event that may enhance cell survival when p21 levels are elevated.

ACKNOWLEDGMENTS

We thank members of the El-Deiry lab for helpful comments. W.S.E.-D. is an Assistant Investigator for the Howard Hughes Medical Institute.

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