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Selective induction of apoptosis through the FADD/Caspase-8 pathway by a p53 C-terminal peptide in human pre-malignant and malignant cells

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A p53 C-terminal peptide (aa 361–382, p53p), fused at its C-terminus to the minimal carrier peptide of *antennapedia* (17 aa, Ant; p53p-Ant), induced rapid apoptosis in human cancer cells, *via* activation of the Fas pathway. We examined p53p-Ant mechanism of action, toxicity in various human normal, non-malignant, pre-malignant and malignant cancer cells and investigated its biophysical characteristics. p53p-Ant selectively induced cell death in only pre-malignant or malignant cells in a p53-dependent manner and was not toxic to normal and non-malignant cells. p53p-Ant was more toxic to the mutant p53 than wild-type p53 phenotype in H1299 lung cancer cells stably expressing human temperature-sensitive p53 mutant 143Ala. Surface plasmon resonance (BIACORE) analysis demonstrated that this peptide had higher binding affinity to mutant p53 as compared to wild-type p53. p53p-Ant induced-cell death had the classical morphological characteristics of apoptosis and had no features of necrosis. The mechanism of cell death by p53p-Ant was through the FADD/caspase-8-dependent pathway without the involvement of the TRAIL pathway, Bcl-2 family and cell cycle changes. Blocking Fas with antibody did not alter the peptide's effect, suggesting that Fas itself did not interact with the peptide. Transfection with a dominant-negative FADD with a deleted N-terminus inhibited p53p-Ant-induced apoptosis. Its mechanism of action is related to the FADD-induced pathway without restoration of other p53 functions. p53p-Ant is a novel anticancer agent with unique selectivity for human cancer cells and could be useful as a prototype for the development of new anti-cancer agents.

Key words: p53 C-terminal peptide; apoptosis; necrosis; BIACORE; TdT assay; FADD (Fas associated death domain)

The p53 tumor suppressor gene is one of the most commonly mutated genes found in human malignancies.[1] More than 50% of human tumors, including breast cancers, are associated with missense mutations or deletions of p53 and most of the missense mutations map to the DNA-binding domain of the protein.[2,3] p53 protein functions in the transcription of growth inhibiting genes, apoptosis, cell cycle arrest and DNA repair.[4–6] p53 is a sequence-specific transcription factor that transactivates a number of genes whose products are involved in cell growth regulation. These include p21 (a cyclin-dependent kinase inhibitor known to arrest the cell cycle mainly in G1/S phase), GADD45 for DNA repair and Bax and Fas/APO-1 for apoptosis.[7–11] p53 induced Fas-FADD binding and transiently sensitized cells to Fas-induced apoptosis.[12]

Programmed cell death depends on conserved structural moieties that transmit and regulate the death signal. This is most evident in death receptors of the tumor necrosis factor receptor (TNF-R) super-family. The intracellular regions of the Fas/CD95 and TNFR1 death receptors have a moderately well conserved NH2-region of about 80 residues that is required for death signalling and has been called the 'death domain' (DD).[13,14] Fas/CD95 induces rapid apoptosis upon binding to Fas ligand through the autocrine/paracrine signalling pathway.[15] When activated, the intracellular death domain in Fas/CD95 binds to FADD/MORT-1 at its C-terminus, which then recruits caspase-8 to FADD. A member of the TNF ligand family identified more recently is TNF-related apoptosis-inducing ligand (TRAIL). TRAIL shows high homology to Fas Ligand and binds to the TRAIL receptor family. TRAIL-induced caspase activation shares many of same caspases involved in Fas/CD95 and TNF-induced cell death mechanisms.[16] FADD/MORT-1 is a cytosolic adaptor protein, which is critical for signalling from Fas/APO-1 and other TNF-R family members.[17] Two protein interaction domains have been identified in FADD/MORT-1. The C-terminal 'death domain' is needed for recruitment of FADD/MORT1 to ligated 'death receptor' and the N-terminal 'death effector domain' mediates oligomerization and activation of caspase-8.[18] Caspase-8 is the first protease activated in the Fas/APO-1 signalling pathway, which initiates downstream activation of caspase-3, -6 and -7 and mitochondrial damage. This activates poly (ADP-ribose) polymerase (PARP) and ICE family proteases, triggering a cascade of apoptotic processes.[19,20]

Apoptosis involves a series of complex biochemical events and is regulated by several pathways, including members of the Bcl-2 family. Bcl-2 is a negative regulator of apoptosis and its' activity is modulated by homodimerization or heterodimerization association with promoters of apoptosis such as Bax and Bad. Bax can be regulated directly by p53 and induced in some cells undergoing p53-mediated apoptosis. Bcl-X_L suppresses apoptosis, whereas Bcl-Xs inhibits Bcl-2 mediated cell survival.[21–23] BID is a novel pro-apoptotic Bcl-2 family protein that is activated by truncation to t-BID by caspase-8 in response to Fas death receptor signals.[24]

The sequence-specific DNA-binding activity of p53 seems to be regulated negatively by its C-terminal 30 aa segment (aa 363–393) and by a N-terminal proline-rich motif located between aa 80–93.[25,26] Synthetic peptides corresponding to the C-terminal domain of p53, aa residues 363–393, bind directly *in vitro* to wild-type (wt) p53. Binding studies with p53 proteins that contain selected deletions suggest that binding of the p53 peptide aa 363–393 to p53 protein requires the presence of both C-terminal aa 363–393 and N-terminal aa 80–93 sequences in the p53 protein.[26] Using conformational energy calculations to compute the lowest energy conformations for the C- and N-terminal regulatory domains, we found that these 2 domains form a unique low energy complex, suggesting that a direct interaction can potentially exist between them.[27]

Previous studies demonstrated that the addition of a chemically modified p53 C-terminal peptide (aa 363–393) restored *in vitro* sequence-specific DNA binding function to mutant p53–273 (Arg to His). Furthermore, intranuclear microinjection of this peptide

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into human colon cancer cells SW480 carrying an endogenous p53–273 His mutant restored transcriptional activation of a p53-responsive reporter construct.[28] A p53 C-terminal peptide (aa 361–382), fused at its C-terminus to the minimal *Antennapedia* (Ant) carrier sequence to facilitate cellular uptake, inhibited growth and induced apoptosis of SW480 cells.[29] Our previous studies indicated that the C-terminal p53 peptide (aa 361–382)-Antennapedia peptide (p53p–Ant) induced selective and rapid apoptosis in human breast cancer cells associated with increased Fas and Fas ligand expression.[27] The mechanism and components of the Fas pathway needed for apoptosis by p53p-Ant were not elucidated. In addition, using human null p53 lung cancer cells H1299 and prostate cancer cells PC-3 stably transfected with the human p53 temperature-sensitive (ts) mutant 143Ala (Val-Ala) as a model, we found Fas-mediated apoptosis occurred only at the wt p53 phenotype at 32.5°C.[30] The mechanism of the induction of Fas-mediated apoptosis by p53p-Ant, its effect upon other human cancer type cells, the differential binding of peptide in cancer cells with mutant *vs*. wt p53 and its effects on normal cells are still unknown. Thus, the mechanism and components of the Fas-mediated apoptotic pathway involved needs to be defined more clearly.

The BIACORE technology system, which is a biosensor system based on surface plasmon resonance (SPR), has been applied widely in studies investigating simple interprotein, protein-DNA and protein-lipid interactions. It can monitor and quantitative the kinetics of binding and dissociation constants between molecules in real-time and without labels.[31–33] We utilized the BIACORE system to quantitate differential binding and dissociation constants between the peptide and mutant and wt p53.

We investigated p53p-Ant's effect on normal, non-malignant, pre-malignant and malignant human cells. We also used human lung cancer cells H1299 expressing human p53 temperature sensitive (TS) mutant 143Ala to study its differential activity and binding to mutant p53 and wt p53 phenotypes with the BIACORE technology. The type of cell death and its effect on p21, Bax, GADD45 and cell cycle and the involvement of caspases were further investigated. To better understand and define the mechanism of p53p-Ant-induced apoptosis, we studied the involvement of Fas, TRAIL and FADD molecules, including dominant negative FADD.

Material and methods

Peptides

p53p, Ant, and p53p-Ant peptides were chemically synthesized by Research Genetics (Huntsville, AL) as described previously.[27] All peptides were HPLC purified to >95% pure. Peptide stocks (4 mM) were prepared in sterile distilled water and stored in aliquots at −80°C. Peptides include: p53p, **N**-GSRAHSSHLKSKKGQSTSRHKK-**C** (22 aa, 361–382 of p53); Ant, KKWKMRRNQFWVKVQRG (17 aa); p53p-AntCONT, **N**-KKGQSTSRKK-WKMRRNQFWVKVQRG-**C** (25 aa); and p53p-Ant, **N**-GSRAHSSHLKSKKGQSTSRHKK-WKMRRNQFWVKVQRG-**C** (37 aa). The fused peptide has 37 aa because we eliminated KK from the NH2 terminus of the Ant sequence because p53p has a KK at the C-terminus. This fused peptide is named p53p-Ant.

Antibodies and reagents

Anti-p21 polyclonal antibody (clone: H-164) was obtained from Santa Cruz (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-human Fas/CD95 monoclonal antibody (clone: DX2) and phycoerythrin (PE)-conjugated mouse anti-human monoclonal TRAIL antibody (clone: RIK-2) were obtained from Pharmingen (San Diego, CA). Anti-human Fas/CD95 monoclonal antibody (clone: CH-11), antagonistic anti-Fas monoclonal antibody (clone: ZB4) and anti-human FADD monoclonal antibody (clone: 1F7) were obtained from MBL International Corporation (MIC, Watertown, MA). Antagonistic anti-TRAIL monoclonal antibody (clone: 2E5) was obtained from Alexis Biochemicals (San Diego, CA). Anti-α-tubulin monoclonal antibody was obtained from Sigma (St. Louis, MO). Caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-AFC) and caspase-9 inhibitor (Z-LEHD-FMK) were obtained from MBL International Corporation (MIC).

Cell lines and tissue culture

The pre-malignant, human colorectal adenoma cell lines RG/C2 (mutant p53/Arg282Trp), BR/C1 (deletion of aa 262–266 of p53) and AA/C1 (wild-type p53) were derived in the laboratory of Drs. C. Paraskeva and A. Williams (University of Bristol, UK). Normal human colon cell line CCD-33Co (wild-type p53), non-malignant human breast cell line MCF10-2A (wild-type p53), malignant human breast cell lines MCF-7 (wild-type p53), MDA-MB-468 (mutant p53), MDA-MB-231 (mutant p53) and MDA-MB-157 (null p53) and human lung cancer cell line H1299 (null p53) were obtained from ATCC. Normal human mammary epithelial cell line HMEC (wild-type p53), non-malignant breast cell line MCF10F (wild-type p53) and its daughter cancer cell line MCFα5 (mutant p53) were kind gifts of Dr. T. Hei (Columbia University, New York, NY). MCF10F (wild-type p53) is a spontaneously derived, immortalized, non-malignant line derived from the normal human breast tissues of a female. MCF10F line was made malignant by gamma radiation *in vitro* and called MCFα5.[34,35] CCD33Co, MDA-MB-468, MDA-MB-231, MCFα5, MCF-7, MDA-MB-157 and H1299 cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine and 100 μg/mL penicillin/streptomycin. AA/C1, RG/C2, BR/C1, HMEC MCF10F and MCF10-2A cell lines were maintained with a 1:1 mixture of DMEM/F12 supplemented with 5% horse serum, 4 mM L-glutamine, 100 μg/mL penicillin/streptomycin, 20 mM HEPES, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin and 20 ng/mL epidermal growth factor.

Plasmid and cell transfectants

Plasmids of pCMV and pCMV/p53-143 were a kind gift of Dr. A. Deisseroth (Sidney Kimmel Cancer Institute, San Diego, CA). DNA fragment corresponding to the NH2 terminus deleted dominant-negative FADD (DN-FADD, aa 80–293) was amplified by RT-PCR mRNA from MDA-MB-468 cells with 5′-forwarding primer containing *Bam*HI site (5′-CCGGATCCGCCACCATGG-ACGACTTCGAGGCGGGG-3′) and 3′-reverse primer containing *Not*I site (5′-AGCGGCCGCTCAGGACGCTTCGGAGGT-3) and then subcloned into a pEGFP-N2 vector (Clontech, Palo Alto, CA). The E1/E3-deleted adenovirus vector pAd/CMV/DN-FADD was constructed and then propagated into 293A cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The virus particle titer was 3×10^8 pu/ml as determined by plaque titration assay in 293A cells. pAd/CMV/DsRed (Invitrogen, Carlsbad, CA) was used as a control. For stable transfection in null p53 H1299 cells, pCMV or pCMV/p53-143 was transfected by using Lipofectamine (GIBCO BRL, Grand Island, NY). The selection of clones was carried out for 2 weeks with 800 μg/ml of G418 (GIBCO BRL, Grand Island, NY). The insertion of the ts p53-143 cDNA plasmid was determined by Western blot. For transient transfection in MDA-MB-468 and H1299 cells, cells were infected with 10 multiplicity of infection (MOI) adenovirus containing pAd/CMV/DsRed (vector) or pAd/CMV/DN-FADD for 24 hr.

Western blot

Cells were untreated or treated with 30 μM p53p-Ant for 3 hr and collected by centrifugation at 2,000*g* for 5 min. Cell lysates were prepared in 1 ml lysis buffer (20 mM Tris CL [pH 7.6], 1 mM EDTA [pH 8.0], 150 mM NaCl, 1% Triton X-100, 10 μg/ml aprotinin, 1 mM benzamidine, 50 μg/ml leupeptin, 10 μg/ml Pepstatin A, and 1 mM phenylmethylsulphonyl fluoride [PMSF]) for 15 min on ice and centrifuged at 10,000*g* for 30 min. Equal amounts of lysates (40 μg) were boiled in SDS sample buffer and loaded on SDS-PAGE. After transferring, immunoreactive prod-

ucts were detected by ECL system (Amersham Pharmacia, Piscataway, NJ). α-Tubulin was used as a control.

Flow cytometric analysis for apoptosis

Cells were treated with 30 μM p53p-Ant for various time points and all cells were collected by centrifugation at 2,000*g* for 5 min. The terminal deoxynucleotidyl transferase (TdT or TUNEL) assay was carried out with a MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan) according to the manufacturer's instructions and analyzed with a FACScater-Plus flow cytometer.

DAPI staining

Cells were treated with various concentrations of p53p-Ant for different periods of time. After collecting all cells by centrifugation at 2,000*g* for 5 min, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 min and then stained with 50 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) at 4°C for 1 hr. Stained cells were examined by using fluorescence microscopy. The number of apoptotic cells with nuclear morphology of apoptosis was scored in at least 400 cells in each sample in three independent experiments, each in triplicate by a blinded reader.

Lactate dehydrogenase release assay for detection of necrosis

Cells were treated with 30 μM p53p-Ant for 1 hr. Lactate dehydrogenase (LDH) released into the surrounding medium was determined according to manufacturer's instructions (Promega, Madison, WI). Results were expressed as a percentage of the maximum LDH release produced by same cell fractionation using repeated freeze-thawing cycles. p53(15)Ant (30 μM), which is a p53 N-terminal peptide (aa 12–26), was used as a positive control for necrosis. We had shown previously that this p53 N-terminal peptide induced cell death only by necrosis without apoptosis.[36,37]

Cell surface Fas and TRAIL analysis

Cells were treated with 30 μM p53p-Ant at indicated time periods. For cell surface Fas or TRAIL analysis, the fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal Fas/CD95 antibody (clone: DX2) and the phycoerythrin (PE)-conjugated mouse anti-human monoclonal TRAIL antibody (clone: RIK-2) were used to determine cell surface Fas or TRAIL receptor expression, respectively, by measuring their fluorescent intensity using FACS-Caliber according to manufacturer's protocol.

Surface plasmon resonance analysis

Binding kinetics were determined by surface plasmon resonance (SPR) using a BIAcore X biosensor system (BIAcore Inc., Piscataway, NJ). The BIACORE SPR system is sensitive to temperature changes. For every 1°C change in system temperature, there is an inverse change of 100 resonance units in the data set. Because the experimental groups analyzed consisted of temperature alterations (32.5°C wt p53 and 37°C mutant p53), we took into account this temperature effect and adjusted the binding data accordingly upon comparison at the 2 different temperatures. p53p-Ant peptide was immobilized on research grade CM5 gold sensor chips (BIAcore Inc.) at a concentration of 50 μg/ml in 10 mM phosphate buffer, pH 6, using the amine coupling kit supplied by the manufacturer. Approximately 1,000 resonance units (RU) of p53p-Ant peptide were immobilized under these conditions, where 1 RU corresponds to an immobilized protein concentration of ~1 pg/mm^2. Unreacted moieties on the surface were blocked with ethanolamine. One of two channels on the instrument was used as a control, with no immobilized peptide and monitored for any background signal contribution. All measurements were carried out in PBS, which contained 10 mM phosphate, pH 7.4, 150 mM NaCl and 3.4 mM EDTA. Analyses were carried out separately on the same chip, at 32.5°C (wt p53 form) and 37°C (mutant p53 form) and at flow rates of 5 or 10 μl/min for the determination of on and off rates and equilibrium binding. H1299 total cell lysate preparations were generally diluted 1:50 in PBS, and the instrumentation and cell lysate prep temperatures were equilibrated for 1 hr at their respective temperatures to stabilize the 2 temperature-sensitive conformations. In all instances, total cell lysate protein concentrations were calculated using the Bradford method, as well as measuring the OD at 280 nm. Surfaces were regenerated to baseline with 10 μl of 50 mM glycine, pH 2.5, in 10 mM PBS, followed by 5 μl of 4 mM cholate, pH 4.5, in the same buffer. BiaEvaluation 3.1 software (BIAcore Inc.) was used to process the raw data using a global fitting model assuming a simple 1:1 bimolecular (Langmurie) binding model ($A + B \leftrightarrow AB$), as well as obtaining kinetic parameters. Excel 2002 (Microsoft, Redmond WA) and Canvas 8 (Deneba Software, Miami, FL) software were used to display the data. The temperature effects on the biosensor data was taken into account and adjusted accordingly for the data obtained.

Results

Toxicity of p53 C-terminal peptide in tumor cell lines

We investigated the selectivity of toxicity of p53p-Ant to various human cell lines, including normal, non-malignant, pre-malignant and malignant cells. Cells were treated with 50 μM p53p alone, Ant alone, p53-AntCONT or p53p-Ant for 24 hr and then assayed by DAPI staining. p53p-Ant, but not p53p alone, Ant alone or p53p-AntCONT, selectively induced cell death only in the mutant p53 pre-malignant human colon cell lines RG/C2 and BR/C1 but was not toxic in the normal colon line CCD33Co and pre-malignant colon line AA/C1 both with wt p53 (Fig. 1*a*). Similar results also obtained by TUNEL assay after treating cells with 50 μM p53p-Ant for 18 hr (Fig. 1*b*). Stable transfectants of human ts p53 mutant 143(Val-Ala) in null p53 human lung adenocarcinoma cells H1299 showed that p53p-Ant was more toxic to mutant than wt p53 cells for induction of apoptosis after exposure to 50 μM p53p-Ant for 18 hr by TUNEL assay. As shown in Figure 1*c*, p53p-Ant was more toxic to H1299/p53-143# 6 cells at 37°C (mutant p53 form) than to its wt p53 form at 32.5°C, 76% *vs*. 46%, respectively. To determine the typical morphological change of apoptotic cells from treatment of p53p-Ant, we used DAPI staining in our study. p53p-Ant, but not p53p-AntCONT, induced cell death at concentrations as low as 10 μM with classical morphological characteristics of apoptosis by fluorescent microscopy, including nuclear condensation and fragmentation after 12 hr treatment in the malignant cell line MDA-MB-468 (Fig. 1*d*). In contrast, there has no cell death in non-malignant MCF10-2A cells when treated with p53p-Ant by the same conditions (Fig. 1*d*). In addition, Table I summarizes the results of 30 μM p53p-Ant exposure in human normal, non-malignant and malignant breast cells, again showing the selectivity of peptide for malignant cells. This also demonstrated that the p53p-Ant requirements for induction of apoptosis were dependent upon presence of endogenous p53 because null p53 H1299 cells were much less sensitive to peptide.

Differential binding of peptide to mutant and wt p53 phenotypes quantified by surface plasmon resonance

A surface plasmon resonance (BIACORE) study was carried out to quantitate p53p-Ant binding to mutant and wt p53. Figure 2*a* depicts the BIACORE sensogram for H1299/ts p53–143 cells at 32.5 and 37°C and shows that p53p-Ant displayed higher binding affinity to the mutant form (37°C). The R_{max} at 37°C was 500 RU, as compared to 420 RU at 32.5°C (wt p53) at 360 sec, indicating overall increased binding saturation capacity for the mutant form (Fig. 2*a*). The association plot (ka), corresponding to the linear slope of binding between 60–360 sec, indicated that the wt p53 form initially bound at a higher rate (steeper positive slope) than mutant p53 form between 60–150 sec (Fig. 2*b*). Between 150–360 sec, however, the mutant form surpassed the wt form (Fig. 2*b*). The dissociation plot (kd) indicated a slower dissociation rate, with a less negative slope for the mutant compared to the wt form between 360–660 sec (Fig. 2*c*). This also suggested tighter binding constants for the mutant p53 form to p53p-Ant.

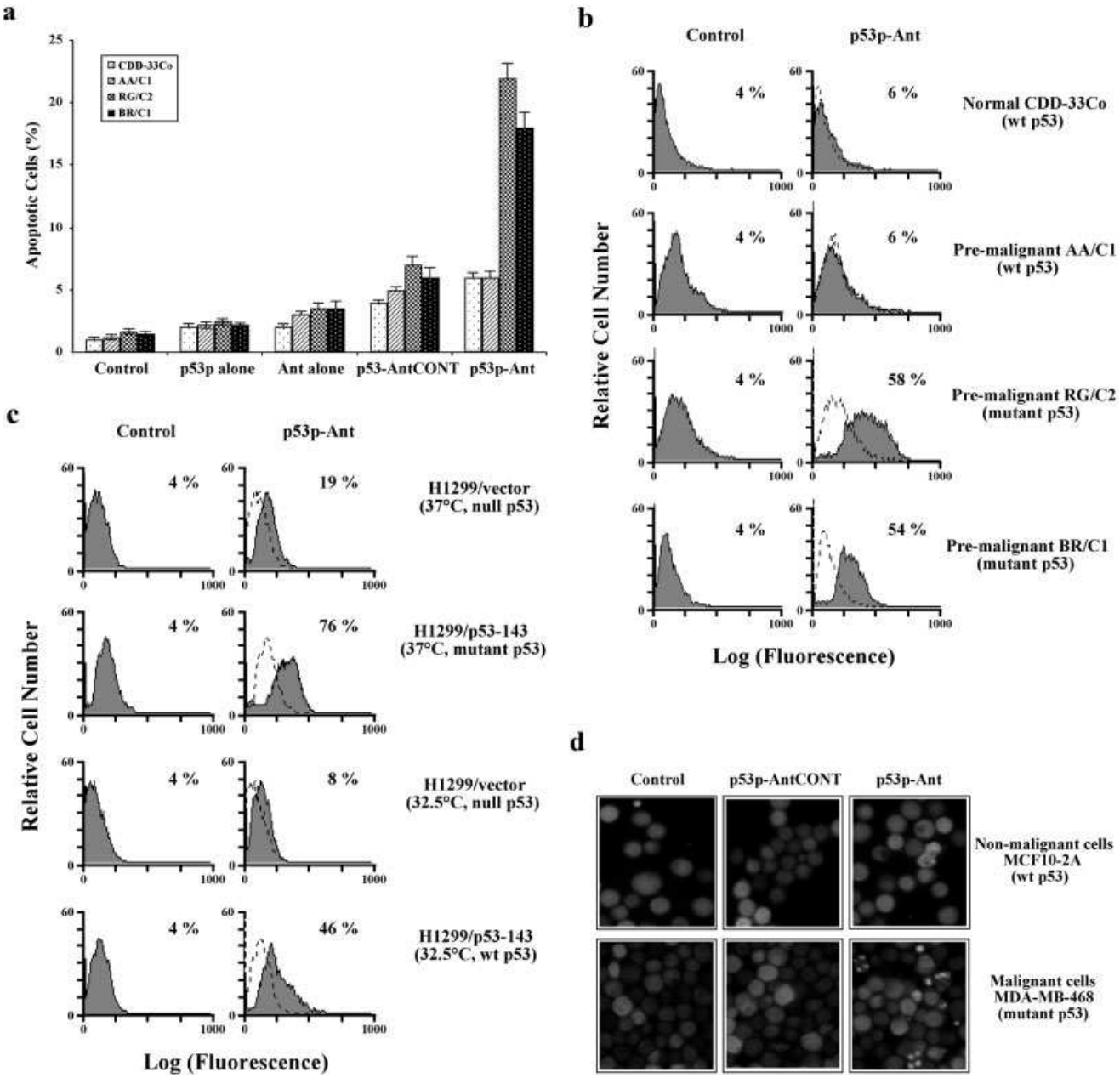

Figure 1 – Selective induction of cell death by p53 C-terminal peptide in normal and pre-malignant human colon cells. (*a*) DAPI staining in normal human colon cells CDD-33Co (wt p53), pre-malignant colorectal adenoma cells AA/C1 (wt p53), RG/C2 (mutant p53) and BR/C1 (mutant p53). Cells were treated with 50 μM p53p alone, Ant alone, p53-AntCONT or p53p-Ant for 24 hr and nuclear morphology was analyzed by DAPI staining. Number of apoptotic cells with nuclear morphology typical of apoptosis were scored in at least 400 cells in each sample by fluorescence microscopy. All microscopy experiments were carried out by a blinded reader to the actual groups. Data represent mean and SD of triplicate experiments. (*b*) TdT (TUNEL) assay in normal human colon cells CDD-33Co (wt p53), pre-malignant colorectal adenoma cells AA/C1 (wt p53), RG/C2 (mutant p53) and BR/C1 (mutant p53). Cells were treated with 50 μM p53p-Ant for 18 hr. Apoptotic cells were determined by TdT assay and representative histograms show relative apoptotic cell numbers. (*c*) Effects of endogenous p53 status upon p53 C-terminal peptide induced apoptosis in H1299 cells with stably transfected human ts p53 mutant 143Ala. Cells were pre-incubated for 16 hr at 37 or 32.5°C and then treated with 50 μM p53p-Ant for an additional 18 hr. Apoptotic cells were determined by TdT (TUNEL) assay and representative histograms show relative apoptotic cell numbers. (*d*) Morphological analysis of apoptotic cells in MCF10-2A (wt p53) and MDA-MB-468 (mutant p53) cells. Cells were treated with 10 μM p53p-AntCONT or p53p-Ant for 12 hr and then nuclear morphology was analyzed by DAPI staining. The typical morphology in 400× magnification.

p53 C-terminal peptide-induced cell death: apoptosis vs. *necrosis*

To further delineate the form of cell death induced by p53p-Ant (apoptosis *vs.* necrosis), we used DAPI staining in time course studies in human breast lines. Cell death started as early as 3 hr and peaked by 12 hr in the malignant mutant p53 cell line MDA-MB-468 but it was not toxic to the non-malignant cell line MCF10-2A (Fig. 3*a*). Similar results also obtained by Trypan blue assays (data not shown). To substantiate that the mechanism of cell death by p53p-Ant was apoptotic and not necrotic, we carried out an early LDH release assay in malignant breast cell lines MCF-7 (wt p53) and MDA-MB-468 (mutant p53). As shown in Figure 3*b*, when

TABLE I – TOXICITY OF p53p-Ant CORRELATES WITH p53 STATUS AND MALIGNANCY IN HUMAN BREAST CELL LINES

Cell line	Cell type	p53 status	Apoptosis (%) by P1 staining	
			Control	p53p-Ant (30 μM 6 hr)
HMEC	Normal	Wild-type	6	9
MCF10-2A	Non-malignant	Wild-type	6	8
MCF10F	Non-malignant	Wild-type	6	7
MDA-MB-468	Malignant	273 Arg>His	6	65
MDA-MB-231	Malignant	280 Arg>Lys	6	32
MCFα5	Malignant	254 Ile>Asp	6	31
MCF-7	Malignant	Wild-type	6	30
MDA-MB-157	Malignant	Null	6	6

compared to the maximal LDH release (MLR), early LDH release into the cytoplasm by 1 hr did not occur after exposure to 30 μM p53p-Ant. The positive control for necrosis, 30 μM p53(15)Ant that is a p53 N-terminal peptide (aa 12–26) shown by us to induce only necrosis without any evidence for apoptosis,[36,37] was carried out simultaneously. The N-terminal p53 peptide induced significant necrosis as evidenced by an early LDH release assay by 1 hr. Also, transmission electron microscopy was carried out at the 2-hr time point and the infrastructure did not show plasma or nuclear membrane perforations from 30 μM p53p-Ant, but the plasma and nuclear membranes displayed perforations after exposure to 30 μM N-terminal p53(15)Ant (data not shown). These data indicated that p53p-Ant mechanism of cell death was purely apoptotic and not necrotic in these 2 cell lines.

Effects of p53 C-terminal peptide on protein targets of p53 and the cell cycle

The effect of p53p-Ant on the expression of proteins induced by p53, including p21, GADD45 and Bax, and the cell cycle were investigated to determine if other wt p53 functions were restored by peptide. There was no increase in baseline p21, GADD45 and Bax expression by 30 μM p53p-Ant treatment in a time course study (0, 3, 6, 12, 24 and 48 hr) in malignant cell lines MDA-MB468 (mutant p53), MDA-MB231 (mutant p53) and MCF-7 (wt p53) (Figure 4 and data not shown). For the cell cycle study, the pattern of cell cycle G1 and G2 peaks did not change in MCF-7 and MDA-MB-468 cells over a 24-hr time course (data not shown).

Effects of p53 C-terminal peptide on caspase activation and Bcl-2 family proteins

To investigate the mechanism of downstream apoptotic induction by p53p-Ant in the Fas signal transduction pathway, we tested its effect on caspase-3 and -8 activation in MDA-MB-468 cells and found the highest caspase-3 and -8 activity appeared 2 hr after treatment with 30 μM p53p-Ant (data not shown). To further confirm this finding, we used the specific caspase-3 inhibitor (Z-DEVD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) at 4 μM to pre-treat MDA-MB-468 cells for 1 hr and then exposed cells to p53p-Ant for 6 hr. Both inhibitors were able to significantly block p53p-Ant-induced apoptosis by TUNEL assays (Fig. 5). In contrast, the caspase-9 inhibitor (Z-LEHD-FMK), which can block the intrinsic pathway of apoptosis induced by the Bcl-2 family of proteins, did not block p53p-Ant-induced cell death. The results confirmed that p53p-Ant-induced apoptosis was through the Fas pathway.

To further determine whether the Bcl-2 family was involved in p53p-Ant-induced apoptosis, we examined the expression of several Bcl-2 family molecules: Bcl-2, Bax, Bcl-X_L, Bak and BID by Western blot analysis in malignant MDA-MB-468 cells treated with 30 μM p53p-Ant for 3 hr. There was no significant change in expression levels of these molecules at 3 hr when apoptosis was evident (data not shown). There were also no changes in the level of heterodimers of Bax or Bcl-2 by immunoprecipitation (IP) assays (data not shown). These experiments further corroborated that the intrinsic pathway of apoptosis (Bcl-2 family proteins) may not be involved in p53p-Ant-induced apoptosis.

p53 C-terminal peptide-induced apoptosis and the Fas signal transduction pathway

To further investigate the mechanism of p53p-Ant-induced cell death through the Fas (extrinsic) apoptosis pathway, we assessed changes in cell surface Fas and TRAIL expression. As shown in Figure 6*a*, MDA-MB-468 cells exhibited a 44% increase in the levels of extracellular Fas protein within 60 min after 30 μM p53p-Ant treatment by flow cytometry. There was no change in TRAIL expression by the same treatment and the controls, p53p and Ant alone, did not have any effect on levels of extracellular Fas expression (data not shown). To determine whether p53p-Ant-induced apoptosis could involve an interaction between Fas and Fas ligand, we pre-incubated the antagonistic anti-Fas antibody ZB4 at 10 μg/ml with MDA-MB-468 cells for 1 hr and then treated with 30 μM p53p-Ant for 6 hr. As shown in Figure 6*b*, antagonistic anti-Fas antibody ZB4 did not block induction of apoptosis by p53p-Ant. For controls, we exposed the cells to the agonistic anti-Fas antibody CH-11 at 50 ng/ml for 24 hr. The ZB4 antagonistic antibody reduced CH-11-induced apoptosis from 34% to 8%. These data suggested the mechanism of p53p-Ant-induced apoptosis was through an increased Fas expression signal transduction pathway independent of Fas–Fas ligand interaction. In addition, experiments with pre-incubation of cycloheximide and actinomycin D for 18 hr did not alter the above results, suggesting that the increased Fas expression was from membrane re-distribution to the extracellular milieu and not from new protein or mRNA Fas synthesis, respectively (data not shown).

Expression of dominant–negative FADD and its effect on p53 C-terminal peptide-induced cell death

To determine whether FADD association to Fas after clustering could be pivotal for p53p-Ant-induced cell death, we modulated FADD expression *via* an Ad5 adenovirus system. Breast MDA-MB-468 and lung H1299/p53-143#6 cells were transfected transiently with adenovirus containing control vector pAd/CMV/DsRed or pAd/CMV/DN-FADD (aa 80–293 of FADD) with 3 different multiplicities of infection (MOI) for 24 hr. The expression of dominant-negative FADD (DN-FADD) was detected by Western blot as shown in Figure 7*a*. Transfectants of MDA-MB-468 were treated with 30 μM p53p-Ant for 6 hr and apoptotic cells were detected by TdT assay. As shown in Figure 7*b*, DN-FADD expression decreased p53p-Ant-induced apoptosis from 41% to 9%. Similar results were also obtained in the H1299/p53-143#6 cells at 37°C (mutant p53 form) exposed to 50 μM p53p-Ant for 18 hr (43% to 15%). These results demonstrated that the N-terminus (aa 1–79) of FADD played a critical role for p53p C-terminal peptide-induced apoptosis in these human cancer cells and the peptide interacted with FADD either directly or indirectly without the need for Fas or Fas ligand.

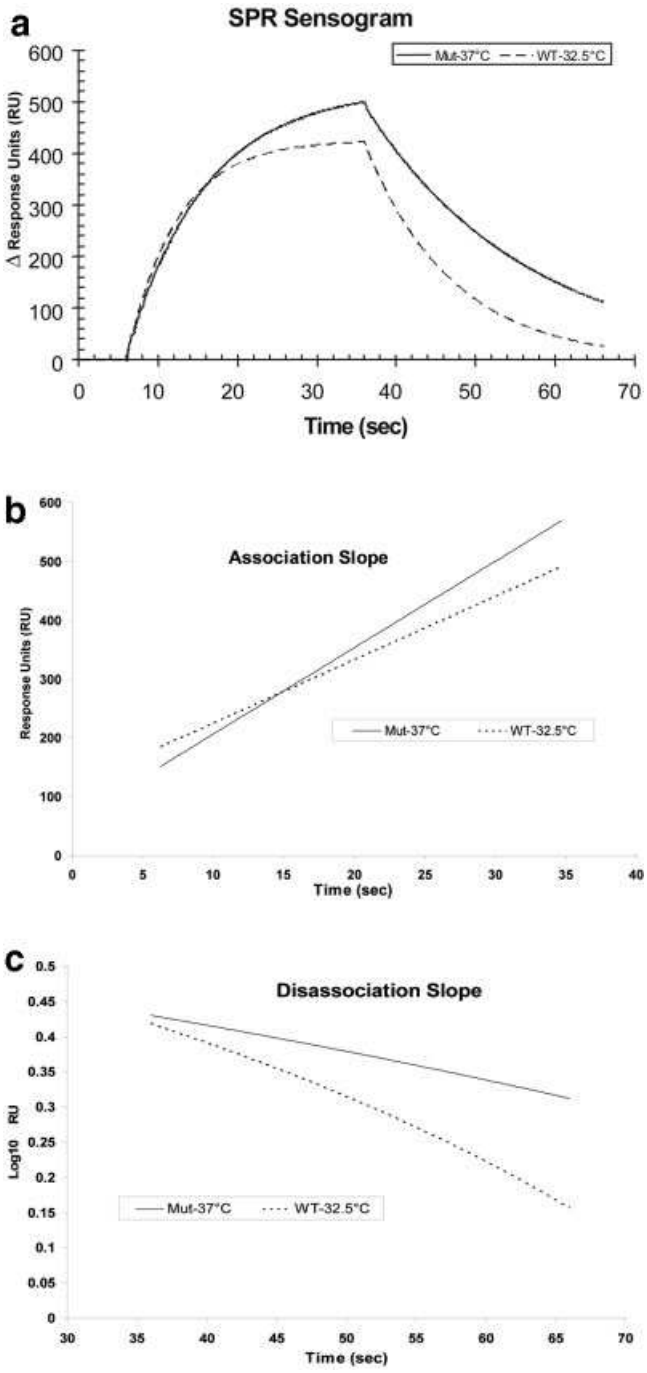

FIGURE 2 – Surface plasmon resonance (SPR) binding assays (BIACORE) of p53p-Ant exposed to H1299/p53-143#6 whole cell lysates. Measurement of binding kinetics are shown between immobilized p53p-Ant at 1 pg/mm^2 and H1299/p53-143 transfectant cell lysates at 32.5°C (wt p53 form; dotted line) and 37°C (mutant p53 form; solid line). Concentration of 10 nM p53p-Ant demonstrated differences in the maximum binding capacity and association/dissociation constants at the two temperatures. SPR were determined by using a BIAcoreX biosensor system. (*a*) Sensogram. (*b*) Association slope. (*c*) Dissociation slope.

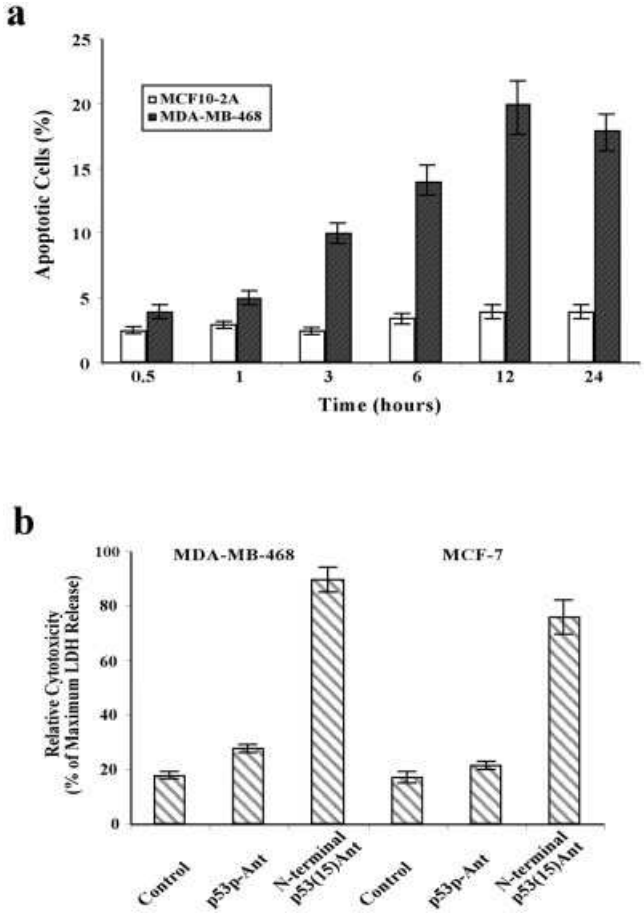

FIGURE 3 – p53 C-terminal peptide-induced cell death: apoptosis *vs.* necrosis. (*A*) DAPI staining in human breast non-malignant cells MCF10-2A (wt p53) and malignant cells MDA-MB-468 (mutant p53). Cells were treated with 30 μM p53p-Ant for the indicated times and nuclear morphology was analyzed by DAPI staining. Number of apoptotic cells with nuclear morphology typical of apoptosis were scored in at least 400 cells in each sample by fluorescence microscopy. All microscopy experiments were carried out by a blinded reader to the actual groups. Data represent mean and SD of triplicate experiments. (*b*) LDH assay in human breast malignant cells MDA-MB-468 (mutant p53) and MCF-7 (wt p53). Cells were untreated or treated with 30 μM p53p-Ant (C-terminal peptide, aa 361–382) or p53(15)Ant (N-terminal peptide, aa 12–26) for 1 hr. LDH released into the surrounding media was determined according to manufacturer's instructions (Promega, Madison, WI). Results are expressed as a percentage of the maximum LDH release, determined by cell fractionation by repeated freeze-thawing. The p53 N-terminal peptide, which only induced necrosis without apoptosis, was used as a positive control for necrosis.[36,37] Data represent mean and SD of triplicate experiments.

Discussion

Our previous work showed that the p53 C-terminal peptide (p53p-Ant) was toxic to 3 malignant breast cells carrying either endogenous p53 mutations or overexpressed wt p53 but was not toxic to malignant breast cells with null p53.[27,38] In this study, we showed that this peptide was not toxic to normal colon and normal, as well as, non-malignant breast cells with endogenous wt p53. The peptide selectively induced apoptosis in pre-malignant colon cells with mutant p53 (Fig. 1*a*, Table I). This is of particular interest because of the paucity of agents that can kill pre-malignant cells. In addition, p53p-Ant was selectively cytotoxic to other tumor types such as prostate, lung, glioma, mesothelioma and colon cancer cell lines in an apparent p53-dependent manner (data not shown). Peptide at 30 μM had only a minor toxic effect on peripheral CD34 positive marrow stem cells in CFU-GEMM assays (10% toxicity), which have normal levels of wt p53 (unpublished data).

To better understand the effects of p53 C-terminal peptide on cancer cells containing different p53 phenotypes, we used ts p53 mutant 143Ala as a model. The results showed p53p-Ant was significantly more toxic to the mutant p53 conformation (at 37°C) than the wt p53 form (at 32.5°C) in H1299 cells stably transfected with a human ts p53 mutant 143Ala (Fig. 1*b*). The surface plasmon resonance (SPR) data suggested a mechanism for the differential cytotoxicity effects observed between mutant and wt forms with p53p-Ant. It may be attributed to increased binding affinity of the p53p-Ant peptide to the mutant p53 form, as compared to the wt form. p53p-Ant initially bound cellular components at the mutant p53 temperature with greater affinity than at the wt p53 temperature, as shown by the higher positive slopes of association (Fig. 2*b*) and greater binding saturation levels indicated by the higher R_{max} (Fig. 2*a*). These results indicated that the mutant p53 form retains more bound complexes with p53 peptide during the binding interaction. More importantly, the dissociation slope for the mutant form exhibited a less negative slope as compared to the wt p53 temperature, suggesting that the mutant p53 form binds peptide more tightly, disassociating less than the wt p53 form (Fig. 2*c*). This could explain why there is more Fas related apoptosis induced in cells with mutant p53 leading to increased restoration of mutant p53 ability for functional redistribution of Fas to the membrane. We are currently studying the binding site(s) of p53p-Ant on p53 by utilizing deletion mutants of p53 and BIACORE analysis.

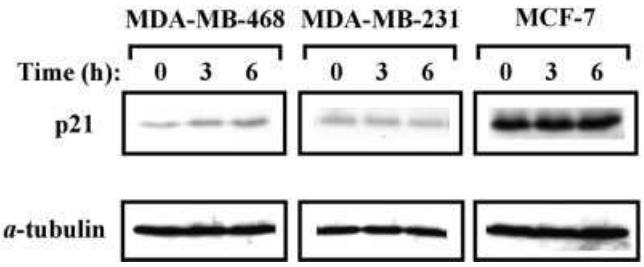

FIGURE 4 – Western blot for p21 expression in human breast malignant cells MDA-MB468 (mutant p53), MDA-MB231 (mutant p53) and MCF7 (wt p53). Cells were treated with 30 μM p53p-Ant for 0, 3 or 6 hr. p21 expression was analyzed by Western blot using anti-p21 polyclonal antibody (clone: H-164). Expression of α-tubulin was used as a control.

We had reported that p53p-Ant activated the Fas/APO-1 signaling transduction pathway. This was associated with activation of the downstream initiator caspase-8 and effector caspase-3 proteases and cleavage of the caspase-3 substrate, PARP.[27] In our study, Fas antagonistic antibody ZB4 did not block apoptosis induced by p53p-Ant peptide but did block Fas mediated apoptosis induced by Fas agonistic antibody CH-11 (Fig. 6). Using anti-Fas antibodies in Western blot analysis, we still did not find increased Fas protein content after exposure to the p53-Ant peptide (data not

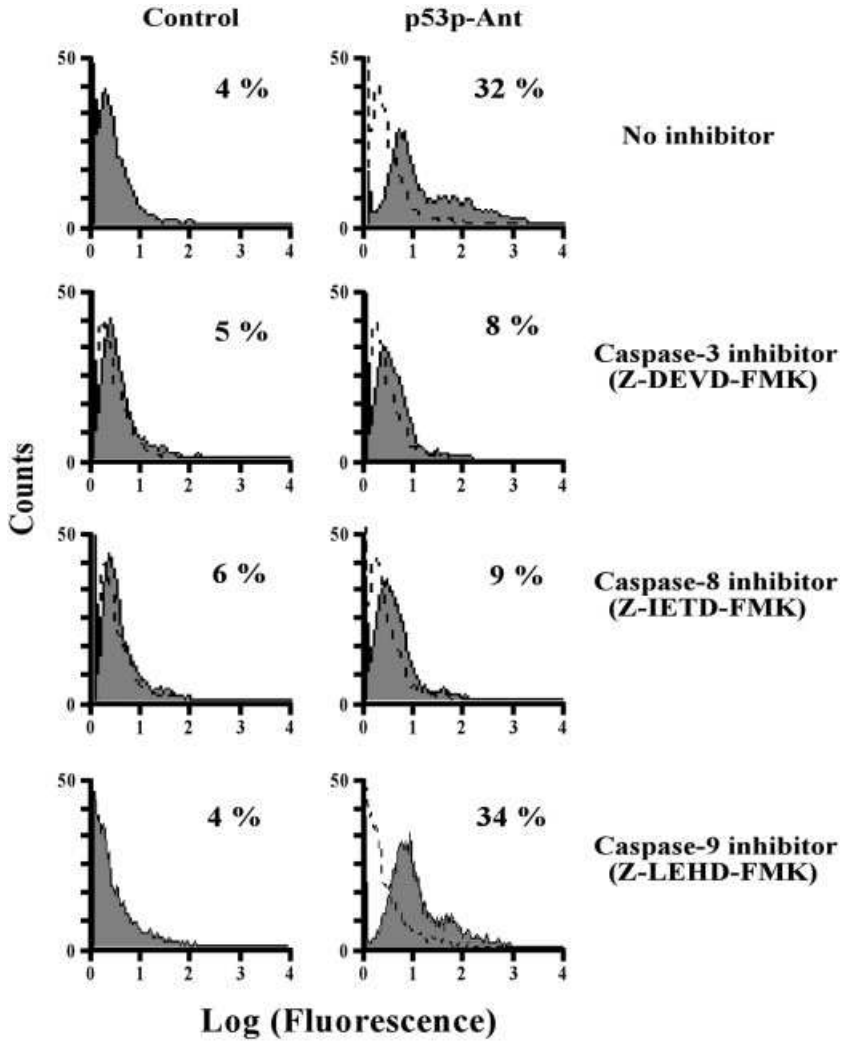

FIGURE 5 – Effect of specific caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) on p53p-Ant-induced apoptosis in human breast malignant cells MDA-MB-468 (mutant p53). Cells were pre-treated with 4 μM inhibitors for 60 min and then treated with 30 μM p53p-Ant for 6 hr. Apoptotic cells were determined by TdT (TUNEL) assay and quantitated by flow cytometry according to the manufacturer's instructions. Representative histograms show relative apoptotic cell numbers.

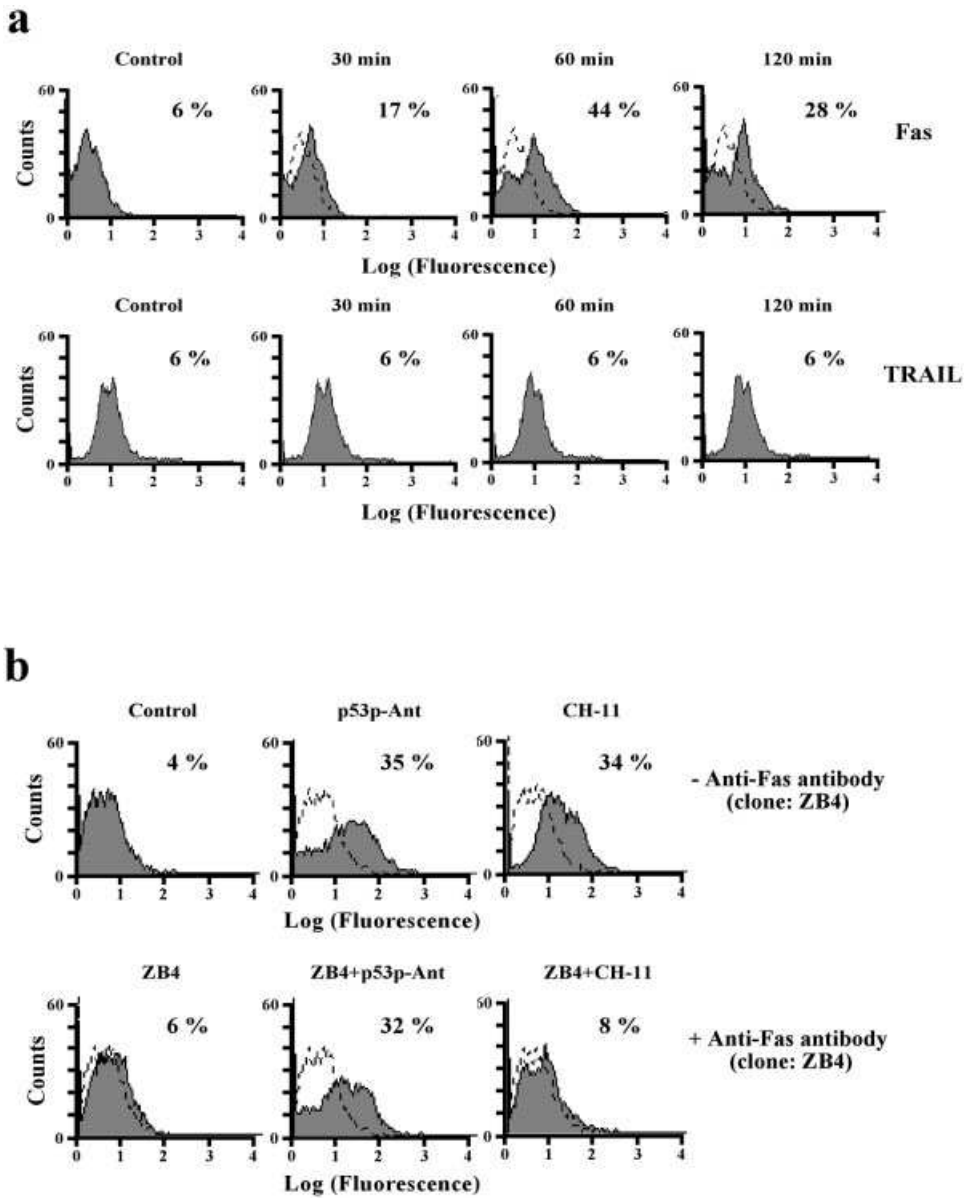

FIGURE 6 – Effect of p53p-Ant on Fas and TRAIL-induced apoptosis pathways. (*a*) Cell surface Fas and TRAIL analysis in MDA-MB-468 cells. Cells were treated with 30 μM p53p-Ant at indicated time periods. Cell surface Fas was determined by FITC-conjugated mouse anti-human monoclonal Fas/CD95 antibody (clone: DX2) and cell surface TRAIL was determined by phycoerythrin (PE)-conjugated mouse anti-human monoclonal TRAIL antibody (clone: RIK-2) using FACS-Caliber according to manufacturer's protocol. (*b*) Effect of antagonistic anti-Fas antibody on p53p-Ant-induced apoptosis in MDA-MB-468 cells. Cells were pre-treated with 10 μg/ml antagonistic anti-Fas antibody ZB4 for 1 hr and then treated with 30 μM p53p-Ant for 6 hr or 50 ng/ml agonistic anti-Fas antibody CH-11 for 24 hr. Apoptotic cells were determined by TdT (TUNEL) assay and quantitated by flow cytometry according to the manufacturer's instructions. Representative histograms show relative apoptotic cell numbers.

shown). This suggested that p53p-Ant-induced apoptosis was not dependent on the interaction of Fas and Fas ligand but may be further downstream of Fas such as FADD. DN-FADD (aa 80–293) clearly blocked p53 C-terminal peptide-induced cell death in 2 different cell lines (Fig. 7*b*), suggesting that FADD played an important role in this mechanism of cell death. We were not able to detect direct Fas/FADD/p53p-Ant binding by IP studies in MDA-MB-468 cells (data not shown). In addition, TRAIL and TNF-R1 were not involved in p53-Ant-induced cell death (Fig. 6 and data not shown).

p53p-Ant did not increase p53 target molecules such as p21, GADD45 and Bax or alter cell cycle characteristics (Fig. 4). These results suggested that p53p-Ant induced only an apoptotic response *via* the extrinsic Fas/FADD pathway without altering the intrinsic apoptosis pathway (Bax) or the cell cycle static response, although the latter 2 are associated with the normal functioning p53 phenotype. Reasons for this dissociative effect are unknown but may be secondary to required conformational changes in p53 that may be different for inducing p21 and GADD45 and re-distribution of Fas to the outer membrane. The peptide's selective induction of Fas-associated apoptosis without p21 induction may provide an added advantage to p53 peptide over whole p53 gene therapy approaches because p21 induction, which can occur from the latter, has been reported to induce drug resistance to cell cycle active agents *via* cell cycle stasis in several tumor cell types.[39,40] In addition, whole p53 gene transfer may hypothetically lead to increased toxicity in normal cells, such as increased sensitivity to chemotherapy drugs by increasing wt p53 levels.

Mitochondrial triggered cell death can occur through several mechanisms including disruption of electron transport, release of proteins that in turn activate caspases and alteration of cellular reduction-oxidation potentials. For example, activation of Fas leads to rapid inactivation of the electron transfer activity and release of cytochrome C from Bcl-2 in the mitochondrial membrane can inhibit cytochrome C release during apoptosis.[41–44] In addition, cytochrome C release from mitochondria and cell death are mediated by BID when cleaved by caspase-8 to t-BID, thus suggesting BID as a downstream component of the Fas pathway.[24] MDA-MB-468 cells treated with p53p-Ant showed no significant changes in expression levels of Bcl-2 family proteins (including BID, Bcl-2, Bax, Bcl-X_L and Bak by

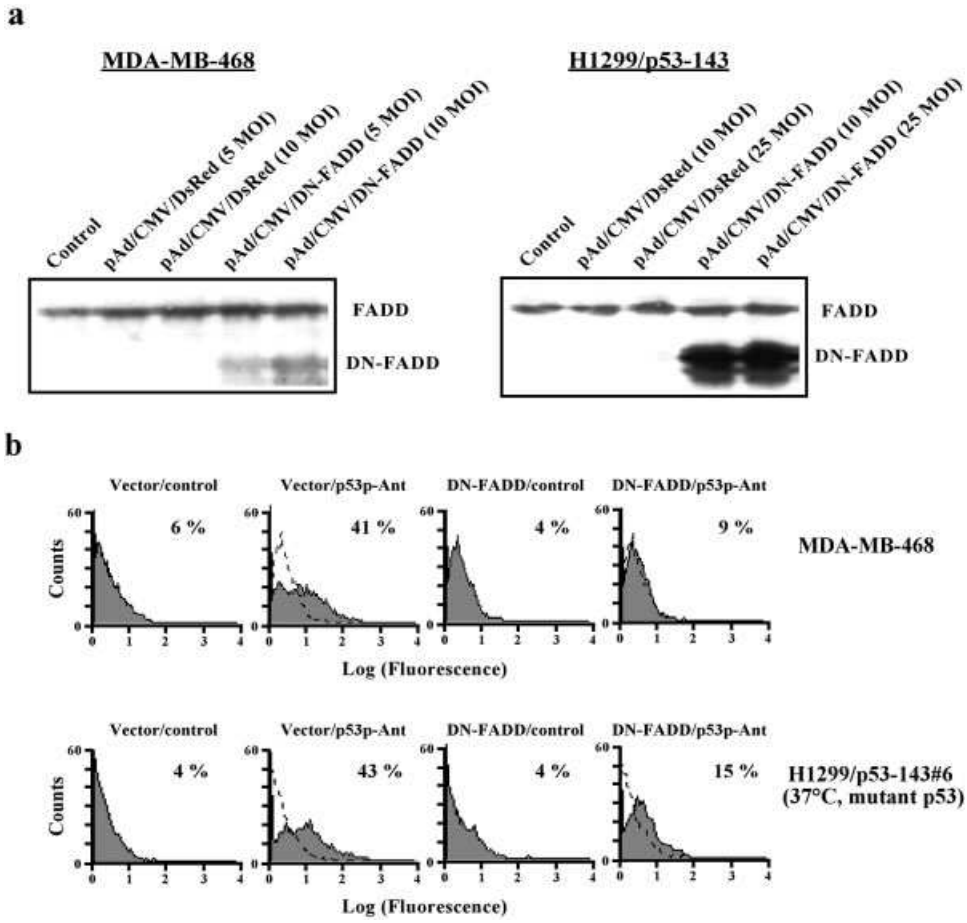

FIGURE 7 – Effect of FADD expression on p53 C-terminal peptide-induced apoptosis. (*a*) Expression of the dominant-negative FADD (DN-FADD, aa 80–293) in MDA-MB-468 breast and H1299/ts p53-143#6 lung cancer cells (at 37°C-mutant p53 form). Cells were infected with 10 MOI adenovirus containing pAd/CMV/DsRed (vector) or pAd/CMV/DN-FADD (DN-FADD) for 24 hr. Expression of DN-FADD protein was detected by Western blot using anti-human FADD monoclonal antibody (clone: 1F7). Expression of α-tubulin was used as a control. (*b*) DN-FADD blocked p53p-Ant-induced apoptosis. Transient transfectants from the 2 cell lines above were treated without or with 30 μM p53p-Ant for 6 hr (MDA-MB-468 cells) or 50 μM p53p-Ant for 18 hr (H1299 cells). Apoptotic cells were determined by TdT (TUNEL) assay and quantitated by flow cytometry according to the manufacturer's instructions. Representative histograms show relative apoptotic cell numbers.

Western blot) and no changes were detected in the heterodimers of Bcl-2/Bax. Using Bcl-2 and Bcl-xL transfectants in human prostate cancer cell line DU-145, which is the sensitive line to p53p-Ant, we did not find that these could block induction of apoptosis by p53-Ant. These results, as well as the lack of caspase-9 activation, suggested that Bcl-2 family proteins (intrinsic apoptosis pathway) were not involved in p53 C-terminal peptide-induced apoptosis. Thus, the peptide acted downstream of Fas, upstream of caspase-8 and through interaction, either direct or indirect, with the N-terminus of FADD. It also has been reported that several anticancer drugs; such as cisplatin, doxorubicin, vinblastine and VP-16 induced cell death through the Fas/FADD pathway in a Fas ligand-independent fashion.[45]

In summary, our study demonstrated that p53 C-terminal peptide fused at its C-terminus to Ant carrier peptide induced only apoptosis and not necrosis. This peptide-mediated apoptosis was selectively toxic to multiple tumor types in mutant p53 pre-malignant and wt and mutant p53 malignant cells, including breast, colon, prostate, lung, glioma and mesothelioma, in a p53-dependent manner. It was not toxic to many normal human cells, however, including breast, colon and marrow stem cell CFU-GEMM colonies. The peptide induced more apoptosis in cells with a mutant p53 phenotype as compared to the wt phenotype. This binding and dissociation of peptide was higher and lower, respectively, for the mutant p53 conformation as compared to wt p53 conformation as verified by the BIACORE study. Our results suggested a possible mechanism whereby the mutant p53 conformation may bind with higher overall affinity to peptide as compared to the wt p53 form, thereby, perhaps allowing more Fas/FADD-induced apoptosis in cells with mutated p53. In addition, cells with mutant p53 usually have more p53 quantitatively than wt p53 cells. Thus, the excess p53 in the mutant p53 cells could serve as more target for the peptide's effect. There was no evidence for involvement of the Bcl-2 pathway, caspase-9, p21 or alteration of cell cycle kinetics, suggesting that the peptide acts mainly through the extrinsic pathway of apoptosis. The mechanism of action was through the Fas pathway and seemed to be downstream of Fas, upstream of caspase-8 and at the N-terminus of FADD, either directly or indirectly. Restoration of mutant p53 and activation of latent wt p53 function through this peptide or through a gene therapy approach may play a future role in cancer therapy for tumors expressing mutant or wt p53.

Acknowledgements

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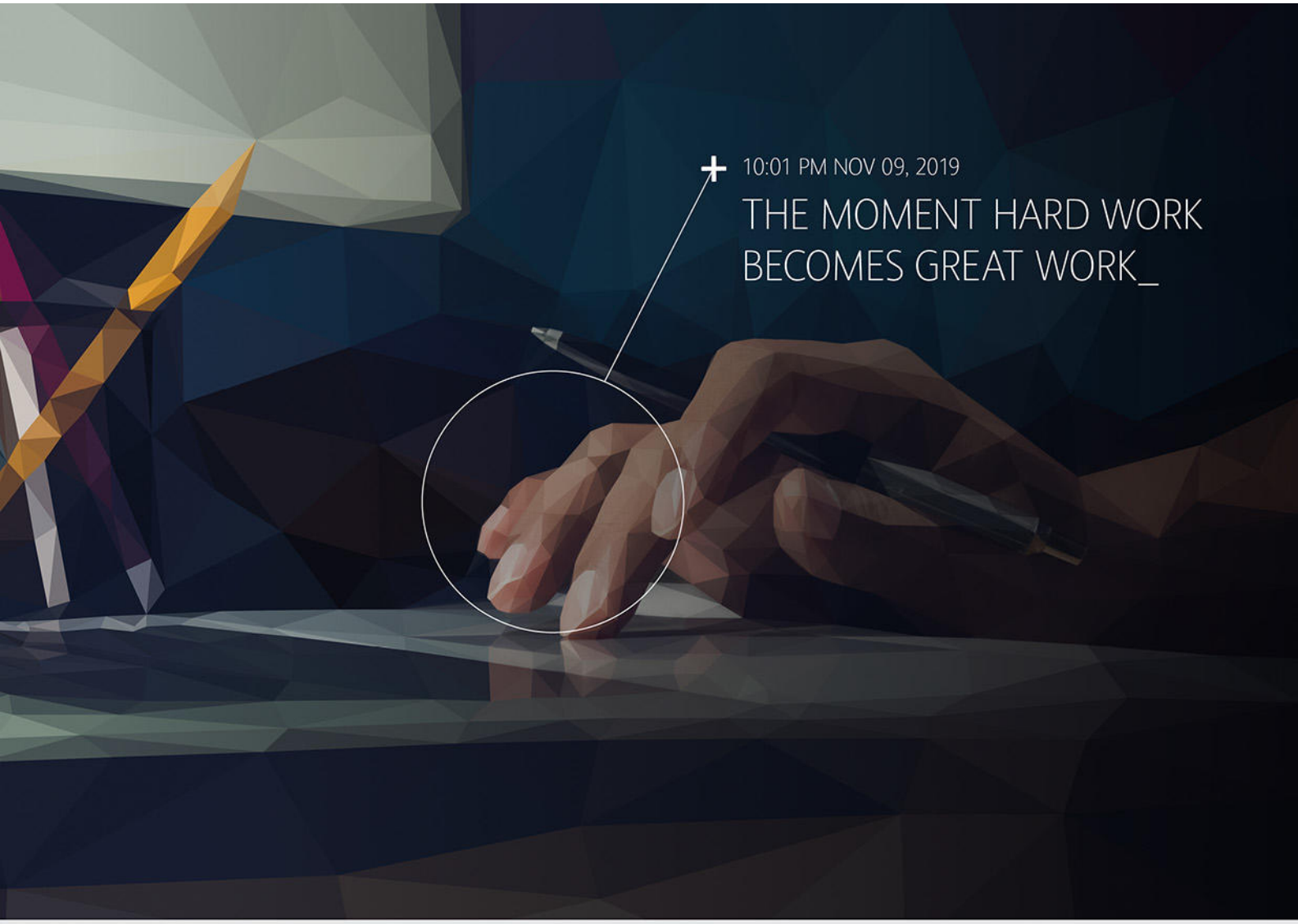

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