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The Penetratin Sequence in the Anticancer PNC-28 Peptide Causes Tumor Cell Necrosis Rather Than Apoptosis of Human Pancreatic Cancer Cells

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Background: PNC-27 and PNC-28 are p53-derived peptides from the human double minute (hdm-2) binding domain attached to penetratin. These peptides induce tumor cell necrosis of cancer cells, but not normal cells. The anticancer activity and mechanism of PNC-28 (p53 aa17–26-penetratin) was specifically studied against human pancreatic cancer.

Methods: MiaPaCa-2 cells were treated with PNC-28. Necrosis was determined by measuring lactate dehydrogenase (LDH) and apoptosis as assayed for measuring elevation of proapoptotic proteins. PNC-29, an unrelated peptide, and hdm-2-binding domain p53 aa12-26 without penetratin (PNC-26) were used as controls. Since there is evidence that penetratin is required for tumor cell necrosis, we tested "naked" p53 peptide without penetratin by transfecting a plasmid that encodes p53 aa17–26 segment of PNC-28 into MiaPaCa-2 and an

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untransformed rat pancreatic acinar cell line, BMRPA1. Time-lapse electron microscopy was employed to further elucidate anticancer mechanism.

Results: Treatment with PNC-28 does not result in the elevation of proapoptotic proteins found in p53-induced apoptosis, but elicits rapid release of LDH, indicative of tumor cell necrosis. Accordingly, we observed membrane pore formation and dose-dependent killing. In direct contrast, transfected MiaPaCa-2 cells underwent apoptosis, and not necrosis, as evidenced by expression of high levels of caspases-3 and 7 and annexin V with background levels of LDH.

Conclusion: These results suggest that PNC-28 may be effective in treating human pancreatic cancer. The penetratin sequence appears to be responsible for the fundamental change in the mechanism of action, inducing rapid necrosis initiated by membrane pore formation. Cancer cell death by apoptosis was observed in the absence of penetratin.

We have developed two peptides, PNC-27 and PNC-28, that contain p53 protein residues 12–26 and 17–26, respectively, attached to a cell membrane-penetrating sequence, called leader or penetratin.[1–3] Although originally conceived to block the binding of p53 to hdm-2 in cancer cells, thereby increasing the half-life of p53 and preventing its ubiquitination and proteosomic degradation, we found that these peptides caused cancer cell death even in cells that lacked p53 expression.[1,3] We further observed that, in cancer cells treated with these peptides, there was no increase in expression of p53-induced proapoptotic proteins such as caspase and Bax.[1–3] Rather, these peptides induced tumor cell necrosis, as evidenced by the rapid release of lactate dehydrogenase (LDH) from treated cancer cells.[2,3] Interestingly, fluorescent-probe-labeled PNC-27 was detected at early stages after treatment in the cell and nuclear membranes.[2] Time-lapse electron microscopy studies later revealed that both peptides induce pore formation in the cell and nuclear membranes, consistent with the peptides' being membrane active.[2] Furthermore, consistent with this activity, we found the three-dimensional structure of PNC-27 by two-dimensional nuclear magnetic resonance (NMR) to be an amphipathic alpha–helix–loop–alpha–helix, a structural motif similar to that of a number of membrane-active peptides.[4,5]

Though these peptides induce tumor cell necrosis among a wide range of different human tumors and cancer cell death of *ras*-transformed pancreatic cancer TUC-3 cells in nude mice,[6] they remarkably have no effect on the growth and viability of a number of normal cell lines. These include rat pancreatic acinar cells, called BMRPA1,[1,3] the normal counterpart of TUC-3 cells, human breast epithelial (MCF-10-2A) cells,[2] and cord blood-derived human stem cells.[1] As previously shown, both peptides appear to induce the killing of cancer but not normal cells by a novel membranolytic mechanism.[2]

In contrast, several studies[7–12] reported p53-dependent apoptosis of treated cancer cells when synthesized peptide sequences targeted to bind to hdm-2 were attached to leader sequences on their amino terminal ends. In one such study,[12] 12 residues from the (h or m)dm-2 binding domain of p53 were synthesized and attached at their amino termini to a TAT leader peptide. This peptide was found to induce apoptosis of uveal melanoma and retinoblastoma cell lines, both containing wild-type p53. Although active against cell lines homozygous for mutant p53, this peptide was not tested against p53-null cells. Interestingly, placement of our penetratin sequence on the amino terminal end of the p53 17–26 peptide resulted in a marked diminution in the cytotoxicity of the peptide to cancer cells (M Kanovsky, MR Pincus, and J Michl, unpublished observations).

Since PNC-27 and PNC-28 both contain p53 sequences known to be involved in the binding of p53 to hdm-2 but induce cancer cell death via membranolysis in a p53-independent manner and display a structural motif of a membrane active peptide that depends on the presence of the penetratin sequence on the carboxyl terminal end of the p53 sequence, we now inquire as to whether penetratin plays an essential role in the membranolytic activity of these peptides. To investigate this question, we introduced the "naked" p53 17–26 peptide, i.e., with no penetratin sequence, into a human pancreatic cancer cell line, MiaPaCa-2, by transfecting a plasmid encoding this peptide into these cells in which peptide expression occurs. We also treated these cells with PNC-28. In both conditions, we measured expression of markers for apoptosis and necrosis to explore whether the naked peptide induces apoptosis in contrast to the same peptide linked to penetratin on its carboxyl terminal end.

MATERIALS AND METHODS

Peptides

The following peptides were synthesized by solid-phase methods and were purified to >95% purity

(Biopeptides Corp, La Jolla, CA): PNC-27 containing residues 12-26 (PPLSQETFSDLWKLL) from the hdm-2 binding domain of p53 and PNC-28 (ETFSDLWKLL) containing residues 17–26 from the hdm-2 binding domain of p53, both attached on their carboxyl terminal ends to the transmembrane-penetrating sequence from the antennapedia sequence, KKWKMRRNQFWVKVQRG, also called penetratin; the control peptide PNC-26, containing only residues 12-26 of p53 and no penetratin and sequence; the control peptide PNC-29, an unrelated peptide from cytochrome P450 (also called X13) (indicated in bold) attached to penetratin (indicated in italics), whose sequence is **MPFSTGKRIML-GE***KKWKMRRNQFWVKVQRG*.

Cells

MiaPaCa-2 cells (human pancreatic cancer cells) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% bovine fetal serum and penicillin/streptomycin (100 U/100 μg/ml) as recommended by the ATCC. BMRPA1 cells (untransformed rat pancreatic acinar cells) were cultured as described previously.[1]

Preparation of Plasmids

DNA encoding the human p53 amino acid residues 17–26 sequence, corresponding to the p53 sequence in PNC-28, was cloned into the mammalian pTracer-SV40 [green-fluorescent protein (GFP)-expressing] expression vector downstream to the SV40 promoter. This vector constitutively expresses a cloned gene (Invitrogen, Carlsbad, CA). Also included in the vector is another expression cassette which is linked in tandem to the SV40-p53 17–26-expressing unit. The second expression cassette contains a cytomegalovirus (CMV) promoter driving the expression of the GFP-Zeocin resistance gene fusion protein. The vector was used to transform TOP10F' chemically competent *E. coli* following the Hanahan method of transformation,[13] and plated on Zeocin-containing agar plates for overnight growth. Eight colonies were then used to inoculate cultures in low-salt Luria broth (LB, 1% bacterial tryptone, 0.5% yeast extract, 0.5% NaCl, and 25 μg/ml Zeocin). Cultures were grown with constant shaking at 200 rpm for 16 h in a 37°C incubator, and plasmids were then extracted by using a Qiagen Spin Miniprep Kit. We synthesized construct sense and antisense strands of the cDNA encoding the p53 17–26 sequence (Invitrogen, Carlsbad, CA). The sense strand sequence was 5′-AG TCGAATTCGCCACC*ATG***GAAACATTTTCAGA CCTATGGAAACTACTT***TGA*GCGGCCGCAGT C-3′). Underlined EcoRI and NotI sites are located in 5′ and 3′ ends of the cDNA, respectively. Start and stop codons are in italics. The p53 17–26 coding sequence is shown in bold. For maximum protein translation in transfected cell lines, we placed the start codon within a Kozak sequence, i.e., GCCACCATG (with ATG being the start codon), which is the optimal context for initiation of translation in vertebrate mRNA.[13] The strands (250 nmol/ml) were annealed in annealing buffer by heating to 95°C, and then cooled to room temperature. The annealed double-stranded p53 17–26-encoding cDNA was then digested with NotI and EcoRI simultaneously. A total of 20 μg pTracer-SV40 was digested with 60 units of NotI and 60 units of EcoRI. Double-digested pTracer-SV40 and p53 17–26-encoding cDNA were then electrophoresed through 0.8% and 2.5 % agarose gel, respectively. Gel bands containing DNA of appropriate size were excised, and DNA content was extracted using the NucleoTrap Gel Extraction Kit (ClonTech, Mountain View, CA). Purified vector and cDNA were ligated with T4 ligase (12 h, 4°C) (New England Biolab, Ipswich, MA). Two microliters of ligation reaction was then dispensed into a vial containing 50 μl One-Shot TOP10F' competent *E. coli* (Invitrogen), and the reaction mixture was incubated on ice for 10 min, heat-shocked to 42°C (30 s), and incubated on ice for another 2 min. A total of 250 μl super optimal broth with catabolite repression (SOC) medium (Invitrogen) was then added to the cells, which were then shaken at 37°C (1 h). This transformation reaction was then diluted 1:100 or 1:10 using SOC medium. A total of 50 μl of each was spread on LB plates containing 12.5 μmol/ml ampicillin that were incubated overnight at 37°C. Eight colonies were randomly chosen to inoculate eight 5 ml overnight LB cultures in the presence of 12.5 μmol/ml ampicillin. Plasmids extracted from each liquid culture were analyzed by automated DNA sequencing using the fluorescence-based dideoxy chain termination reaction (Genewiz, North Brunswick, NJ). We found that they all contained the correct p53 17–26 cDNA reading frame associated with a stop codon and a start codon embedded in the Kozak sequence. This construct plasmid is termed p53 17–26-V ([expression] vector).

We followed precisely the same procedure for preparation of a plasmid encoding a scrambled p53

peptide sequence (residues 12–26) as follows: 5′-AG TCGAATTCGCCACC*ATG***TGGGACCTGACACTA CCCAAACAGCTTCTACCTTCAAGTTTCGAA***TGA* GCGGCCGCAGTC-3′, with start, stop codons, and restriction enzyme sites denoted as above. This plasmid construct is called p53-12-26-scrm-V

Transfection into Cancer Cells

We transfected p53 17–26-V plasmid and the two control plasmids [the p53 12-26-scrm-V vector and the second vector encoding GFP only, called the empty vector (EV)] into MiaPaCa-2 and untransformed BMRPA1 cells. We then evaluated these cells for viability, and expression of: p53 protein, p53 17–26 peptide, caspase, annexin binding to phosphatidyl serine, and LDH. Twenty-four hours prior to transfection 5×10^5 cells were seeded in antibiotic-free medium into each well in a six-well tissue culture dish (TCD) and allowed to adhere overnight. To three wells, 0.8 μg of p53 17–26-V plasmid were added. To the other three wells, 0.8 μg of empty vector or p53-12-26-scrm-V vector encoding control peptide were added. To each of these wells, Lipofectamine 2000 transfection agent (Qiagen) was added such that the ratio of plasmid DNA (in μg) to Lipofectamine 2000 (in μl) was 1:2.[14] This ratio was determined in preliminary experiments described in the next paragraph. Transfections were performed in serum- and antibiotic-free culture medium at 37°C for 4 h, at which time the incubation was continued in complete medium, containing 10% fetal bovine serum (FBS) and penicillin and streptomycin (100 U/100 μg/ml). After another 4–5 h, the cells were washed, followed by reincubation in fresh complete medium. Transfection efficiency was measured by examining the frequency of GFP-expressing cells in the total cell population 12 h post transfection by using a Zeiss LSM 410 confocal laser scanning microscope.

For each cell line studied the effective ratio of DNA to Lipofectamine 2000 reagent was verified in preliminary experiments using a checkerboard assay. In these experiments, transfection efficiency was established by titration of various concentrations of DNA in the presence of increasing concentrations of Lipofectamine 2000 on cells that had been seeded onto glass coverslips. After transfection, the GFP-positive (GFP+) cells on the coverslips were quantitated by counting under a UV Light Zeiss epifluorescence microscope in 3–5 consecutive fields counting 200–400 cells. These preliminary experiments helped to establish the cell density, the amount of DNA, and the ratio of DNA to Lipofectamine 2000 to be used, and the time for transfection to proceed.

Expression of the p53 17–26 Peptide

For protein analyses, and detection of apoptosis, 2×10^6 cells were seeded into 10-cm-diameter TCDs and transfected with DNA/Lipofectamine 2000 proportionally adjusted to the increased area. When the cell density reached 90–100%, the cells of the experimental and sham-transfected group were detached using trypsin and plated into four new TCDs in which they were allowed to grow in complete medium. At defined time points cells were released from adherence with 10 mM ethylene diamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS) and were lysed in lysing buffer [1% Triton X-100 in 0.05 M Tris-HCl (pH 8.0), 0.15 mM NaCl, 0.02% Na azide, 0.01 mg/ml phenylmethylsulfonylfluoride (PMSF), and 0.001 mg/ml Aprotinin]. Protein equivalents of 10^6 cells, i.e., ~30 μg/lane, were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris-HCl gels and, in some experiments, 16% Tricine Peptide Gels (Biorad, Hercules, CA) to detect PNC-28 (approximately 3,104 Da) and p53 17–26 (approximately 1,500 Da). The separated proteins were then electrophoretically transferred to nitrocellulose membranes followed by immunoblotting with the mAb DO-1 to p53 AA residues 11–25, and with mAb B-2 to GFP (each at 1–2.5 μg/ml blotting buffer), respectively.[2] After washing unreacted mAbs from the membranes, the membranes were incubated (1 h) with a second enzyme-labeled antibody from the enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ) to detect the presence of p53 and p53 17–26 peptide. In preliminary experiments it was noted that identification of p53 protein was easily possible within 30–90 s of exposure while clearly identifiable binding of mAbDO-1 to p53 17–26 peptide took much longer time. The membrane was therefore cut across the 17 kDa marker (kaleidoscope polypeptide standard), to allow for the differential exposures. In addition, a time course of GFP expression was performed in both MiaPaCa-2 and BMRPA1cells, which established that, during the time period 48–96 h post transfection, the cells showed the highest levels of GFP expression. Semiquantitation of immunoblotting results was performed by measuring luminosity of bands in a single scanned developed X-ray film, using the histogram option of Adobe Photoshop version 5.5. The background level was ascertained by measuring the aver-

age luminosity of five areas of the film outside the blotting region. The opacity of each band was calculated by using the equation: opacity = 255 – luminosity – background.[15]

Incubation of MiaPaCa-2 Cells with PNC-28

Duplicate sets of 6×10^6 MiaPaCa-2 cells were incubated with different concentrations of PNC-28, i.e., 5, 10, 20, 40, 80, and 160 μmol/ml. Duplicate control experiments were also performed in which 6×10^6 MiaPaCa-2 cells were incubated with the control peptide, PNC-29, present at a concentration of 75 μmol/ml. All incubations were carried out using a protocol identical to that described previously.[1] After the cells had been allowed to adhere to the tissue culture dish (TCD) for 24 h the medium was removed from each TCD, and new medium containing the same or no peptide concentration was added. Medium from each TCD was removed every 24 h, and fresh medium with its respective peptide at the appropriate concentration was added. Cells were inspected daily for changes in cell growth, morphology, and viability. At the end of each day over a 5-day period, duplicate cell counts were performed for each incubation using the trypan blue exclusion method. In addition, cell viability was also determined by using the 3-[4,5-dimethylthiazol-2yl]-2,5 diphenyl tetrazolium bromide (MTT) assay according to the manufacturers' instructions (Promega Corporation, Madison, WI, USA).

Incubation of Peptides with BMRPA1 Cells

These cells are untransformed rat pancreatic acinar cells.[1] Duplicate 5-day incubations were performed on 6×10^6 cells in three circumstances: with no peptide, with PNC-28 at 75 μmol/ml, and with PNC-29 at 75 μmol/ml. Cells were followed for viability and morphology over this time period. At the end of 5 days, cell counts were performed using the trypan blue exclusion method.

Immunocytochemistry for Annexin V-Binding to Phosphatidyl Serine

To determine whether any of the transfected plasmids induced apoptosis, we evaluated whether the cells contained phosphatidyl serine in the inner cell membrane, identified as binding to annexin-V, as a marker for apoptosis.[15] Cells (5×10^5) were seeded in six-well TCDs 24 h prior to transfection in antibiotic-free medium. Cells were then either transfected with p53 17–26-V, p53 12–26-scrm-V, EV or were left untreated. At predetermined time points post transfection, the cells were released using 0.5× trypsin–EDTA, collected, and processed as described in the manufacturer's instructions of the Annexin V-Biotin Apoptosis Detection Kit (CalBioChem, La Jolla, CA). The stained cells were resuspended in antifade (Molecular Probes, OR), mounted on glass slides under a glass coverslip, and evaluated for red (TRITC) and green (GFP) fluorescence by using confocal microscopy as described above.

Evaluation of Cells Treated with PNC-28 Peptide for Caspase as a Marker for Apoptosis and LDH Release as a Marker for Necrosis

Cells from culture plates at 18, 44, 66, and 90 h time points were lysed in situ in cell lysis buffer [1% Triton X-100 in 0.05 M Tris-HCl (pH 8.0), 0.15 mM NaCl, 0.02% Na azide, 0.1 mg/ml phenylmethylsulfonylfluoride (PMSF), and 0.001 mg/ml Aprotinin]. Lysates were subjected to 10% SDS-PAGE followed by electrotransfer to nitrocellulose and immunoblotting with antibodies to GFP and p53 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody-labeled proteins were identified by chemiluminescence using the ECL methodology (Amersham).[1] Assays for elevated caspase expression were performed using the Clontech (Palo Alto, CA) for caspase (CPP32) activity.[2] As a positive control for the caspase activity assay, we incubated Mia-PaCa-2 cells with tumor necrosis factor (TNF) (Sigma, St. Louis, MO) at a concentration of 10 ng/ml for 24 h. In addition, to detect if significant cell necrosis occurred we used the CytoTox96 assay (Promega, Madison, WI) for LDH released into the cell culture medium as we performed on several breast cancer cell lines.[2]

Electron Microscopy of MiaPaCa-2 Cells Treated with PNC-28

Time-lapse electron microscopy (EM) was used to examine the ultrastructural features of cell death. MiaPaCa-2 cells were grown for 24 h on Thermanox coverslips (Lux Scientific), and then treated with 25 μmol PNC-28 for 1 and 15 min, along with a corresponding control group without peptide. The cells were washed with PBS solution and then fixed with 2.5% glutaraldehyde–PBS. The fixed cultures were rinsed in a 0.1 M phosphate buffer (pH 7.3), post fixed in 2% (0.08 m) osmium tetroxide–PBS (pH 7.3), dehydrated in a graded series of ethanol and

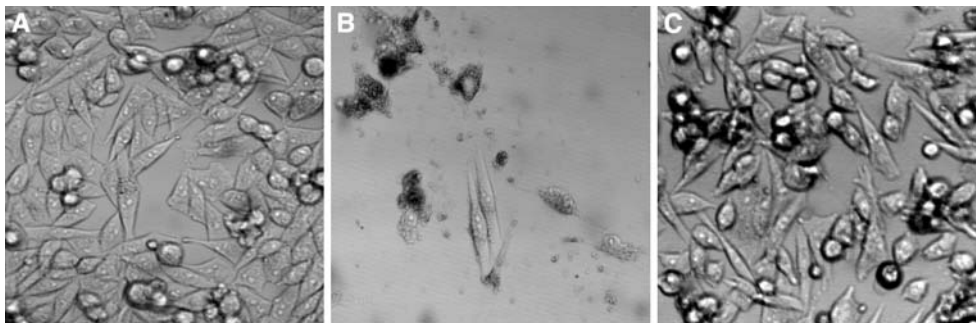

FIG. 1. PNC-28 is cytotoxic to MiaPaCa-2 cells. (**A**) Untreated MiaPaCa-2 cells incubated for 24 h. (**B**) MiaPaCa-2 cells treated for 24 h with 75 μmol/ml of PNC-28. (**C**) MiaPaCa-2 cells treated with 75 μmol/ml PNC-29 negative control peptide for 48 h.

propylene oxide, and embedded in Epon 812. Sections were cut at 700 Å, stained with uranyl acetate and lead citrate, and examined by using a Jeol JEM 1010 electron microscope.

Blotting of Mia-PaCa-2 Cell Lysates for p53 and waf^{p21}, a Target for Activated p53

Cell lysates were prepared as described in the preceding paragraph and were subjected to immunoblotting with either DO-1 antibody described in the section above for expression of the p53 17–26 peptide, a (Ab-6) monoclonal anti-p53 antibody (Calbiochem) or with polyclonal anti-waf^{p21} antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:2000 dilution) using a procedure identical to that described in the same section above. For controls, we blotted for actin using anti-actin polyclonal antibody (Santa Cruz Biotechnology).

Statistical Analysis

Analysis of growth inhibition and markers for necrosis and apoptosis were analyzed by a two-tailed Mann–Whitney nonparametric test or a two-tailed Student *T*-test where appropriate. A *P*-value of less than 0.05 was considered significant.

RESULTS

PNC-28, but Not Negative Control Peptide PNC-29, Is Cytotoxic to MiaPaCa-2 Cells

We incubated PNC-28 with 6×10^6 Mia-PaCa-2 cells for 5 days at concentrations ranging from 0 to 160 μmol/ml. The anticancer effect on MiaPaCa-2 cells incubated with PNC-28 is shown in Fig. 1A–C.

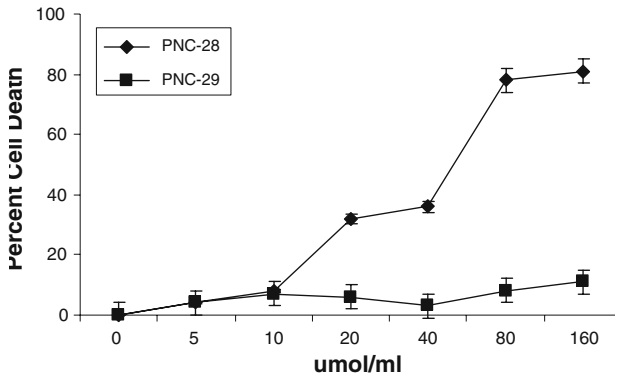

FIG. 2. PNC-28 (diamonds) is cytotoxic to MiaPaCa-2 cells in a dose-dependent manner over a dose range from 20 μmol/ml (35% cell death) to 80 μmol/ml (>80 % cell death) at 48 h. The effect of the control PNC-29 (squares) is also shown. The effective dose range for PNC-28 at 48 h from 20 to 80 μmol/ml is strongly statistically significant ($P < 0.001$).

Figure 1A demonstrates untreated MiaPaCa-2 cells that are not contact-inhibited and are spindle-shaped, many becoming multinucleated. After 24 h of treatment with 75 μmol/ml PNC-28, these cells appear necrotic demonstrated by membrane blebbing and disruption, forming cell clumps coalescing into aggregates of cellular debris (Fig. 1B). At 48 h, there was near 100% cell death as measured by trypan blue dye uptake. In contrast, negative control peptide PNC-29 at 75 μmol/ml had no effect on cellular growth, morphology, and viability (Fig. 1C).

Figure 2 shows inhibition of proliferation after 48 h of peptide treatment. The effective peptide dose ranged between 20 and 75 μmol/ml. It should be noted that doses of PNC-28 between 20 and 75 μmol/ml induced virtually 100% cell death; the times required for cell killing decreased as the dose increased. For example, 75 μmol/ml induced near-total cell death in 48 h while 31.25 μmol/ml induced similar cell death in 4 days, and 20 μmol/ml induced cell death in 1 week.

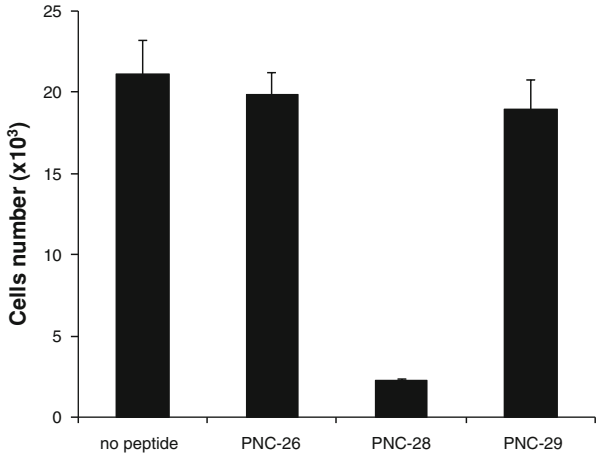

FIG. 3. Carboxyl terminal attached penetratin to residues 17–26 (PNC-28) is required for cytotoxicity: MiaPaCa-2 cell death following treatment with 75 μmol/ml of PNC-28 (condition 3), PNC-26 (condition 2), and PNC-29 (condition 4) after 48 h of treatment.

Figure 3 summarizes the cytotoxic effects on MiaPaCa-2 cells of PNC-28 but not control peptides, including PNC-29 and the "naked" p53 17–26 peptide (PNC-26), which cannot traverse the cell membrane since it lacks the penetratin sequence.[3] Since PNC-29 contains the penetratin, but not the p53, sequence and has no effect on cell growth, the penetratin sequence itself does not induce the observed cytotoxicity.

As a further control, we repeated our original experiment[1] by incubating 75 μmol/ml PNC-28 with untransformed BMRPA1 acinar cells[1] and with the untransformed breast epithelial cell line, MCF-10-2A,[2] and found no growth inhibition or cytotoxicity (data not shown; see previous report[1,2]). These results suggest that PNC-28 is lethal specifically to cancer cells and does not interfere with normal cell growth, as we concluded in our previous studies.[1,2]

Markers for Necrosis and Apoptosis in MiaPaCa-2 Cells Treated with PNC-28

In previous studies, we found that PNC-28 induced cancer cell death in a variety of human cancer cells[1,2] by inducing tumor cell necrosis rather than apoptosis.[2] This was manifested in baseline expressions of caspase but high levels of LDH within 24 h in the medium, indicative of membrane lysis. We therefore investigated the expression of LDH and caspase in MiaPaCa-2 cells treated with PNC-28. Figure 4A shows that LDH activity is elevated in cells treated with PNC-28 (condition 2) almost to the same extent as cells that were lysed with lysis buffer (condition 1). On the other hand, only baseline levels of LDH were found for cells treated with control PNC-29 peptide.

That PNC-28 induced tumor cell necrosis is supported by electron micrographs of MiaPaCa-2 cells treated with this peptide in a study that is identical to the one we performed on breast cancer cells.[2] As shown in Fig. 4B, MiaPaCa-2 cells treated with PNC-28 (left panel) exhibit lysis of their plasma membranes, as we found previously for breast cancer cell lines,[2] in contrast to untreated cells (right panel) that have their plasma membranes intact. This pattern is characteristic of tumor cell necrosis.[2]

In contrast, as can be seen in Fig. 4C, only baseline levels of caspase were expressed in MiaPaCa-2 cells treated with PNC-28, identical to the level expressed in cells treated with control peptide PNC-29. This finding confirms our conclusion that PNC-28 does not induce apoptosis. Thus PNC-28 induces tumor cell necrosis in MiaPaCa-2 cells as we found for other cancer cell lines.[1,2]

The results shown in Fig. 4A–C were obtained using 25 μmol/ml PNC-28. As shown in Fig. 4D, the same results were obtained with all doses of peptide that we used over the 20–75 μmol/ml range, i.e., early release of LDH but only background levels of caspase, suggesting that tumor cell necrosis is induced at all concentrations of PNC-28 and that this mechanism of induction of cell death is not dependent on peptide concentration.

Transfection of MiaPaCa-2 Cells with a Plasmid That Encodes the p53 17–26 Sequence

Results of Transfection of MiaPaCa-2 and BMRPA1 Cells

Two hours after transfection we performed cell counts on slides using light microscopy and then counted the number of cells exhibiting green fluorescence from GFP. On this basis we found that 30–45% of the cells expressed GFP, as summarized in Table 1. The highest transfection rates were found for MiaPaCa-2 cells, whether transfected with empty vector (EV) or p53 17–26-encoding vector (p53 17–26-V).

Morphological Examination of Transfected Cells, as Visualized by Inverted Light Microscopy

In our initial set of transfection experiments, cells were observed by light microscopy beginning 18 h after transfection. MiaPaCa-2 cells transfected with p53 17–26-V were visibly hypertrophic, with many showing membrane blebbing and some being necrotic. In contrast, BMRPA1 cells transfected in the

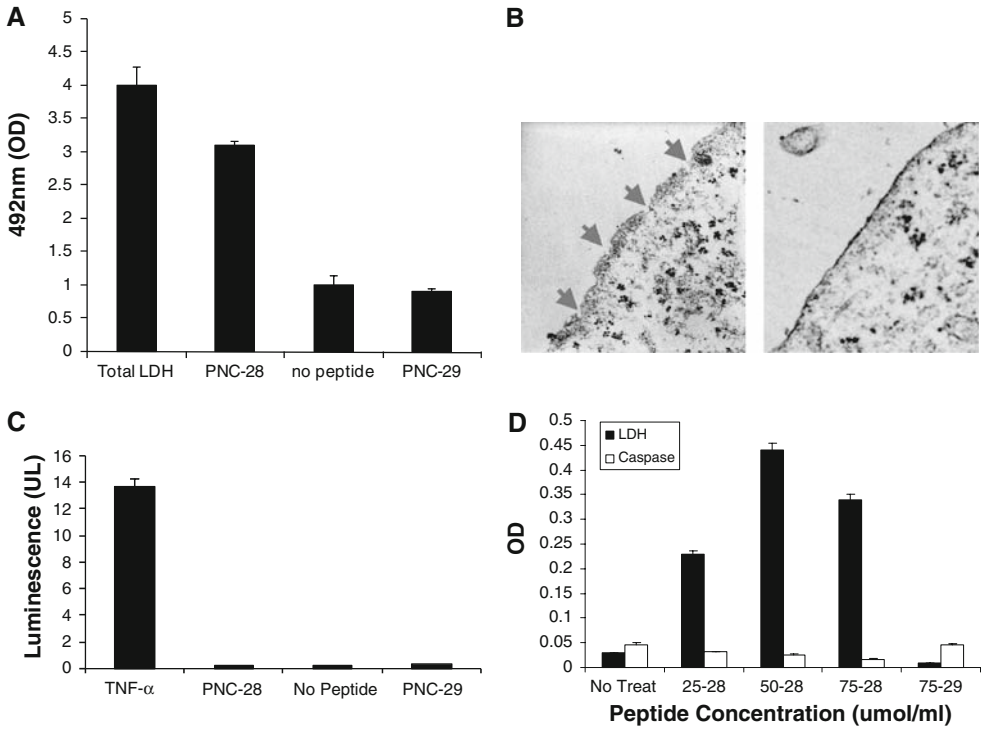

FIG. 4. (**A**) PNC-28 induces cellular death by necrosis in MiaPaCa-2 cells. LDH activity (measured as absorbance at 492 nm) was recorded for MiaPaCa-2 cells incubated with 25 μmol/ml of PNC-28 (condition 2), no peptide (condition 3), and PNC-29 (condition 4) at 24 h. Maximal LDH release is shown after treatment with lysis buffer (condition 1). (**B**) Electron micrographs of MiaPaCa-2 cells that were untreated (right panel) or treated (left panel) with 25 μmol/ml PNC-28 for 15 min. The arrows in the left panel point to gaps or holes in the cell membrane induced by PNC-28. (**C**) PNC-28-induced cell death not caused by apoptosis. Caspase 3 activity recorded for MiaPaCa-2 cells incubated with 25 μmol/ml of PNC-28 (condition 2), no peptide (condition 3), and PNC-29 (condition 4) at 24 h. Maximal caspase release is shown after treatment with TNF-α (condition 1) known to induce apoptosis. (**D**) PNC-28 induces cell death by causing tumor cell necrosis and not apoptosis over its entire effective concentration range. At each dose, both LDH and caspase activity were measured after incubation with PNC-28 after 24 h. For the points on the abscissa, two numbers are separated by a dash. The first number refers to the concentration of peptide; the second number refers to the particular peptide, e.g., "28" refers to PNC-28.

TABLE 1. *Efficiency of transfection of plasmids into MIA-PaCa-2 and BMRPA1 cells*

Cell line	Plasmid	GFP (%)
MIA-PaCa-2	Empty vector (EV)	43
MIA-PaCa-2	p53 17–26-V	45
MIA-PaCa-2	P53 17–26-scrm-V	37
BMRPA1	P53 17–26-V	30

same way as MiaPaCa-2 by either EV or p53 17–26-V showed little alteration in morphology. This observation was confirmed 90 h post transfection, as by this time point the cells maintained there normal polygonal epithelial cell morphology, identical to that of untreated cells.

Cell Viability Post Transfection

Figure 5 shows the effect of transfection of the plasmid p53 17–26-V encoding the p53 17–26 peptide on cell viability for MiaPaCa-2 and untransformed BMRPA1 cells. As can be seen in this figure, within 48 h, transfection of this plasmid induces 60% cell death (condition 1) while transfection of p53 17–26-scrm-V control plasmid results in a much lower level of cell death, i.e., 20%, as shown in condition 2. This is a baseline level since this is the level of cell death we observed for untransformed BMRPA1 cells transfected with the same control vector (condition 4). In condition 3 of this figure, it can be seen that expressed p53 17–26 peptide has a much less pronounced effect on BMRPA1 cells, resulting in the same baseline level of cell death seen in control plasmid-transfected BMRPA1 cells (condition 4). Thus expression of the peptide induces cell death in cancer, but not in untransformed, cells.

Effects of the p53 17–26 Peptide on MiaPaCa-2 Cells

We lysed MiaPaCa-2 cells expressing GFP that had been transfected with EV or p53 17–26-V and blotted for p53, waf^{p21}, a protein that is induced by a p53-

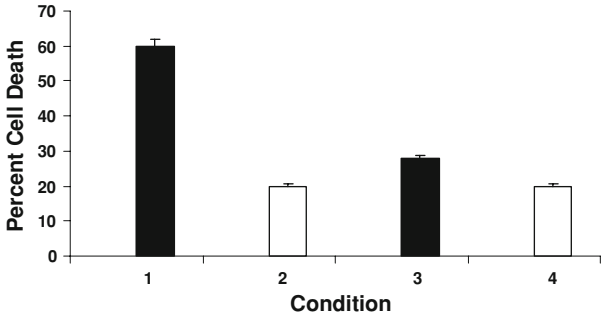

FIG. 5. Cell death (number of dead cells divided by total cell count) as measured by trypan blue dye exclusion in MiaPaCa-2 and BMRPA1 cells transfected with either p53 17–26-V or control p53 17–26-scrm-V plasmid, 48 h post-transfection. Condition 1: MiaPaCa-2 cells transfected with p53 17–26-V (black); condition 2: MiaPaCa-2 cells transfected with p53 17–26-scrm-V (white); condition 3: BMRPA1 cells transfected with p53 17–26-V (black); condition 4, BMRPA1 cells transfected with p53 17–26-scrm-V (white).

dependent pathway, and the p53 17–26 peptide itself. In these experiments, we used the DO-1 anti-p53 antibody that recognizes a determinant that contains residues 17–26 of p53.[2] In addition, we measured caspase activity in these cells. For comparison, we performed the same set of experiments on Mia-PaCa-2 cells treated with 75 μmol/ml of PNC-28. As can be seen in Fig. 6, at zero time (left side of Fig. 6, labeled "0 time") after transfection or incubation with PNC-28, peptide, p53, waf^{p21}, and caspase activity are all expressed at baseline levels. At times when cell death was near 100% (at 96 h for transfected cells and at 48 h for PNC-28-treated cells) peptide levels were found to be high in both transfected and PNC-28-treated cells (Fig. 6, right side, labeled "100% cell death"). However, in the transfected cells, it can be seen that there are elevated levels of p53, waf^{p21}, and caspase activity (labeled "transfection" on the right side of the figure) that are not observed in the PNC-28-treated cells (labeled "PNC-28" on the right side of the figure). For controls, we blotted for actin and found that the levels were the same for all four conditions in Fig. 6 (not shown). These results suggest that the p53 17–26 peptide induces increased intracellular expression of p53 protein with consequent apoptosis of MiaPaCa-2 cells, as evidenced by the concomitant increased expression of waf^{p21} that does not occur in cells treated with PNC-28.

Interestingly, when we blotted lysates from untransformed BMRPA1 cells transfected either with EV or with p53 17–26-V, we found only low levels of expression of p53. In addition, we found that there was only a low level of expression of p53 17–26 peptide in p53 17–26-V-transfected cells (results not shown). Since GFP was expressed at high levels in these cells, it appears that expressed peptide is unstable in these untransformed cells.

Expressed p53 17–26 Peptide Induces Apoptosis, Not Necrosis, of MiaPaCa-2 Cells

Since expression of p53 and waf^{p21} was elevated in cells transfected with p53 17–26-V to much higher levels than in cells transfected with control vector, we concluded that the peptide was inducing apoptosis, in contrast to its counterpart PNC-28 peptide discussed above. We sought further confirmation of peptide-induced apoptosis. In the early stages of apoptosis, phosphatidyl serine (PS), normally present in the inner leaflet of the bilayer membrane of intact cells, is found on the external plasma membrane of cells undergoing apoptosis. Annexin V binds PS and can be located by a probe that carries the red fluorescent TRITC probe. Consequently, cells that had been transfected approximately 48 h earlier were processed for staining with annexin V–biotin followed by streptavidin–TRITC. Figure 7 shows the confocal microscopic results for MiaPaCa-2 cells transfected with control vector (upper left), showing green fluorescent cells with no red staining, and cells transfected with p53 17–26-V that show green fluorescent cells with strong red staining for PS. On the right-hand side of the figure are the results for BMRPA1 control cells that have been transfected with control vector (upper right) or p53 17–26-V (lower right). As can be seen in Fig. 7, neither panel is positive for PS in the normal control cells. As discussed above, expression of p53 17–26 peptide is low in this cell line, possibly causing the absence of signs of apoptosis. We discuss this finding further in the "Discussion" below. Thus the p53 17–26 peptide induces apoptosis in the cancer cell line only.

Caspase and LDH in Transfected MiaPaCa-2 Cells

We further assayed p53 17–26-V-transfected cells for caspase and LDH release to compare our results with those from MiaPaCa-2 cells treated with PNC-28 peptide (Fig. 4). As shown in Fig. 8A (condition 3), caspase activity is over fourfold higher in these treated cells than in untreated cells (condition 2) and has the same fold-increase over that in cells treated with PNC-28 peptide and control PNC-29 peptide. In contrast, as shown in Fig. 8B, LDH release from these transfected cells (condition 3) is at the baseline level found for untreated cells (condition 2) and is about fivefold lower than release from cells treated with PNC-28 (condition 4). Thus presence of the penetratin sequence in PNC-28 results in a change in

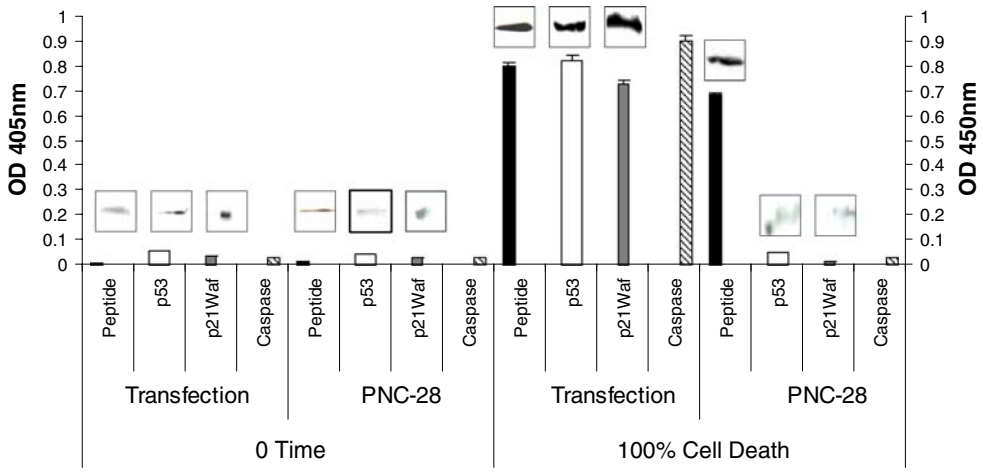

FIG. 6. Effects of expression of the p53 17–26 peptide, following transfection of its expression vector into MiaPaCa-2 cells, on expression of p53 and waf^{p21}, a cell cycle inhibitor protein induced by activated wild-type p53, as a function of time, measured by immunoblotting and on caspase activity. Peptide expression in cells was measured by blotting for the peptide with the anti-p53 monoclonal antibody DO-1 that recognizes the p53 17–26 sequence expressed by the plasmid. For comparison, the effects of incubating PNC-28 with MIA-PaCa-2 cells on induction of these proteins and on caspase activity are also shown. Intracellular PNC-28 level was determined using the same DO-1 antibody. The left ordinate shows the absorbance results for the caspase activity assay while the right ordinate shows the band intensity for each Western blot; the actual immunoblots are shown above each bar graph for the two proteins, p53 and waf^{p21}, and each peptide (p53 17–26 and PNC-28). The left side of the figure shows the results for zero time, after Mia-PaCa-2 cells were transfected with the plasmid (labeled ''transfection'') and immediately after PNC-28 was added to the incubation medium (labeled ''PNC-28''). The right side of the figure shows the results after close to 100% of the cells were killed by the plasmid-expressed peptide (labeled ''transfection'') and by PNC-28 (labeled ''PNC-28''). Actin controls were the same for all four conditions (not shown).

the mechanism of action of the p53 17–26 peptide; without penetratin, the peptide induces apoptosis, whereas with penetratin on its carboxyl terminal end, the peptide induces tumor cell necrosis.

DISCUSSION

Prior Evidence That the Leader Sequence in PNC-27 and PNC-28 Is Required for Induction of Tumor Cell Necrosis

The purpose of this study was to define the role of the leader sequence in PNC-28 in inducing tumor cell necrosis. In our previous studies with this peptide and its parent peptide, PNC-27, we found that both peptides induced tumor cell necrosis, not apoptosis, and caused necrosis even in cancer cells in which p53 protein was absent.[1–3] These findings suggested that both peptides exerted their effects independently of p53 activation. They contrasted with the results of studies in which similar p53 sequences were attached to penetratin on their amino terminal ends and induced p53-dependent tumor cell apoptosis, not necrosis.[7–11] This results from the binding of these peptides to hdm-2 in place of the p53 protein; these peptides are not themselves ubiquitinated since the sites for p53 ubiquitination lie outside this domain.[16]

Further evidence that the presence of the leader sequence on the carboxyl terminal end of PNC-27 and 28 is essential for its induction of tumor cell necrosis is the three-dimensional structure of PNC-27. We found that this is a highly amphipathic alpha–helix–loop–alpha–helix structure that is found in membrane-active peptides.[4] Disruption of this structure, as may occur by placement of the leader sequence on the amino terminal end of the peptide, would be expected to change the activity of the peptide. We found that the p53 17–26 peptide containing the penetratin sequence on its amino terminus, called reverse or r-PNC-28, has much lower activity in cell killing than does PNC-28 itself (M. Kanovsky, J. Michl, and M.R. Pincus, unpublished observations). These findings support the conclusion that the leader sequence is critical to the activity of the peptide but leaves open the question as to whether the ''naked'' p53 peptide itself can induce necrosis or apoptosis of cancer cells.

Cells Transfected with pTracer-SV40 Plasmid Encoding p53 17–26 ''Naked'' Peptide Express This Peptide

To define the role of the leader sequence definitively, we sought to determine the effects of the p53 17–26 peptide itself on tumor cell growth, i.e., whether even without the leader sequence, it could induce

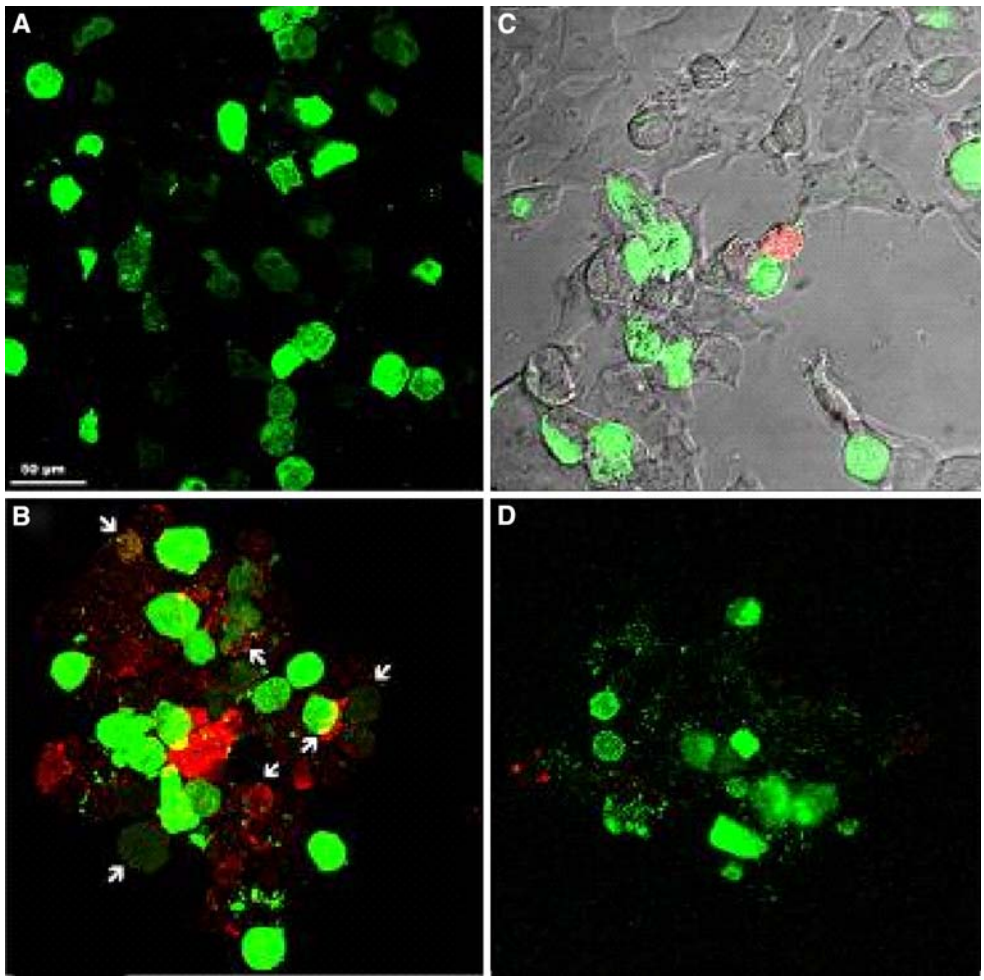

FIG. 7. Transfection of p53 17–26 vector induces apoptosis in MiaPaCa-2 but not BMRPA1 cells. Confocal microscopy demonstrating green fluorescence following transfection of control vectors into MiaPaCa-2 and BMRPA1 cells (**A** and **C**). Annexin V binding to phosphatidyl serine (red staining) detected in p53 17–26-V-treated MiaPaCa-2 cells (**B**) but not in 17–26-V-treated BMRPA1 cells (**D**).

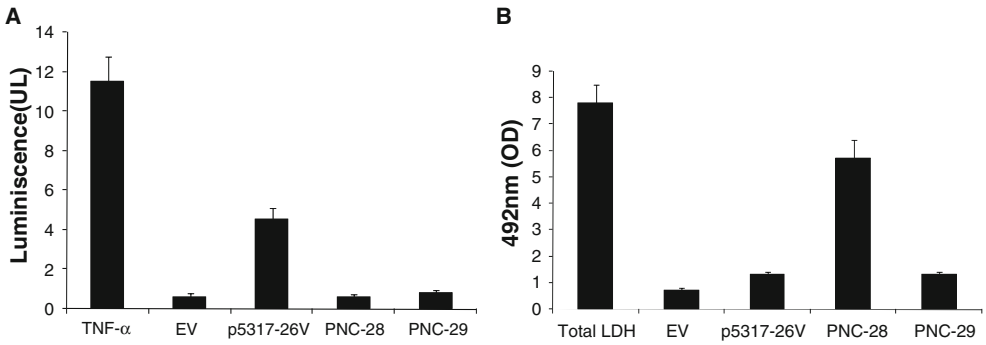

FIG. 8. (**A**) p53 17–26 induces cellular death by apoptosis. Caspase 3, 7 activity recorded (luminescence, as labeled on the ordinate) for MiaPaCa-2 cells transfected with p53 17–26-V (condition 3), empty vector (condition 2), PNC-28 (condition 4), and PNC-29 (condition 5). Maximal caspase release is shown after treatment with TNF-α (condition 1). (**B**) p53 17–26-induced cell death does not cause necrosis. LDH activity recorded for MiaPaCa-2 cells transfected with p53 17–26-V (condition 3), empty vector (condition 2), PNC-28 (condition 4), and PNC-29 (condition 5). Maximal LDH release is shown after treatment with lysis buffer (condition 1).

tumor cell necrosis. To accomplish this goal, we introduced the p53 peptide into MiaPaCa-2 cells via transfection using the pTracer-SV40 plasmid that constitutively expressed this peptide. We then determined the expression of markers for apoptosis and necrosis in the transfected cells and compared the levels of these markers with those found in MiaPaCa-2 cells treated with PNC-28.

As can be seen in Table 1, transfection efficiencies were relatively high. MiaPaCa-2 cells transfected with the p53-peptide-expressing plasmid expressed high levels of the p53 peptide and expressed high levels of GFP as revealed by Western blots over this time period. By 90 h, when at least two-thirds of the cells were killed, peptide expression decreased to barely detectable levels while GFP levels also decreased significantly. This phenomenon may have been caused by cell death and release of proteases causing peptide degradation. On the other hand, BMRPA1 cells transfected with the same peptide-encoding plasmid expressed much lower levels of this peptide. Since these cells expressed high levels of GFP, which is expressed under the same promoter, it is not likely that p53 peptide was not also being synthesized in these cells. One possible explanation for this observation is the status of hdm-2. Recently, we found that this protein is expressed at barely detectable levels in untransformed cells, including BMRPA1 cells, but is expressed at high levels in transformed cells (V. Adler, W. Bowne, K. Sookraj, E. Yazdi, J. Michl, and M.R. Pincus, manuscript in preparation). If binding of the small p53 decapeptide to hdm-2 blocks its degradation in cells, absence of hdm-2 may make the peptide susceptible to intracellular proteases, resulting in its degradation.

The p53 17–26 Peptide Induces Apoptosis of Cancer Cells

As summarized in Fig. 6, expression of the p53 17–26 peptide in MiaPaCa-2 cells induces increasing expression of p53 with concomitant increasing expression of waf^{p21} over a time period in which cell death increases. These results are compatible with binding of the peptide to hdm-2, blocking the binding of p53 to this protein, resulting in prolongation of the half-life of p53. This would result in its increased expression in MiaPaCa-2 cells, allowing it to cause apoptosis, explaining the increasingly elevated levels of waf^{p21}. As shown in Fig. 8A, activity of caspase, a marker for p53-dependent apoptosis, is elevated to almost five times the background level (condition 2) in p53 17–26 peptide-expressing MiaPaCa-2 cells (condition 3). Likewise, it is elevated above background level in cells incubated with PNC-28 (condition 4). Also, in virtually all MiaPaCa-2 cells treated with p53 17–26 peptide-expressing cells, there was strong expression of annexin-V-binding phosphatidyl serine in the membranes of transfected MiaPaCa-2 cells, a known early phenomenon in apoptosis,[15] but not in MiaPaCa-2 cells transfected with empty vector (Fig. 7). These results suggest that the naked p53 17–26 peptide causes cancer cell death by inducing apoptosis. Furthermore, the p53 peptide-expressing MiaPaCa-2 cells do not release LDH in 24 h (condition 3, Fig. 8B) as would be expected if the peptide induced tumor cell necrosis. In contrast, treatment of MiaPaCa-2 cells with PNC-28 resulted in high levels of LDH (condition 2, Fig. 4A and condition 4 in Fig. 8B) over this time course that began as early as several minutes after treatment, confirming that the peptide induces tumor cell necrosis and not apoptosis. This effect is independent of the concentration of PNC-28, suggesting that its mechanism of action does not change in a concentration-related manner. Our finding that expression of the p53 17–26 peptide in a cancer cell line induces apoptosis is in agreement with the results of prior studies that showed that similar peptides from the hdm-2 binding domain of p53 likewise induce apoptosis.[7–12]

Overall, our results strongly suggest that the p53 17–26 peptide induces tumor cell apoptosis and not necrosis. On the other hand, presence of the leader sequence on the carboxyl terminal end of the p53 17–26 peptide plays a critical role in changing the mechanism of cell killing of this peptide from apoptosis to tumor cell necrosis.

The p53 17–26 Peptide Induces Apoptosis in Tumor but Not Normal Cells

As shown in Fig. 5, transfection of empty vector in MiaPaCa-2 cells causes a low level of cell death. However, as shown in Fig. 7, transfection of empty vector into MiaPaCa-2 cells causes no exposure of phosphatidyl serine as revealed by absence of annexin V binding, suggesting that the transfection does not induce apoptosis. On the other hand, transfection of the vector inducing expression of the p53 17–26 sequence into these cells induces higher rates of cell death (Fig. 5). In all of these cells, as shown in Fig. 7, there is strong annexin binding, suggesting that these cells are all undergoing apoptosis.

In contrast, transfection of the p53 peptide-expressing vector into untransformed BMRPA1 cells does not result in an increase in cell death over the

background (Fig. 7). Furthermore, neither transfection of empty vector or of p53-peptide-expressing vector results in any exposure of annexin-binding phosphatidyl serine (Fig. 8). These findings may be due, at least partly, to the low level of expression of the p53 17–26 peptide. Nonetheless, the p53-peptide-encoding plasmid does not induce apoptosis in untransformed BMRPA1 cells. Thus, like PNC-27 and PNC-28 peptides, the p53 17–26 peptide appears to be selective for killing cancer but not untransformed cells.

In summary, the penetratin or leader sequence on the carboxyl terminal end of PNC-28 causes it to induce tumor cell necrosis. Removal of the penetratin sequence from the p53 17–26 peptide in cancer cells results likewise in cytotoxicity to these cells except in this case, by apoptosis of the tumor cells. Thus the presence of in this case, the leader sequence on the carboxyl terminal end of p53 peptide results in a fundamental change in the mechanism of action by which the peptide causes tumor cell death. Like the full peptide, PNC-28, the naked p53 17–26 peptide appears to be selective for the induction of apoptosis of cancer cells but not of untransformed cells. This may be due in part to low levels of expression of the peptide in untransformed cells that express low levels of hdm-2 that may shield expressed peptide from protease degradation. This finding implies that introduction of the naked peptide into cells can induce tumor cell apoptosis while leaving normal cells unaffected.

In the future, additional studies must place emphasis on further defining the selective anticancer mechanism of these peptides as well as their cancer-specific target(s). These results underscore the importance of elucidating the p53–hdm-2 interaction in the cancer cell, whereby we may gain a better understanding of the manner in which this complex potentiates possible crosstalk between necrotic and apoptotic pathways that may lead to novel approaches for directed therapy. As a result, such peptide therapeutics could deliver selective cytotoxic small molecules to cancer cells, targeting pathways inducing necrosis, apoptosis or both.

ACKNOWLEDGEMENTS

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