SHORT COMMUNICATION

Induction of the apoptosis-promoting protein Bak by perillyl alcohol in pancreatic ductal adenocarcinoma relative to untransformed ductal epithelial cells

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Perillyl alcohol has antitumor activity toward pancreas and other cancers with low toxicity. Here, we have investigated the mechanism of action responsible for the differential sensitivity of malignant versus non-malignant pancreatic cells to the drug. We report that the rate of apoptosis is over 6-fold higher in perillyl alcohol-treated pancreatic adenocarcinoma cells than in untreated cells, and that the effect of perillyl alcohol on pancreatic tumor cells is significantly greater than its effect on non-malignant pancreatic ductal cells. Moreover, the perillyl alcohol-induced increase in apoptosis in all of the pancreatic tumor cells is associated with a 2- to 8-fold increase in the expression of the proapoptotic protein Bak, but Bak expression is not affected by perillyl alcohol in non-malignant cells. Thus, the antitumor activity of perillyl alcohol toward pancreatic cancers may be due to preferential stimulation of Bak-induced apoptosis in malignant versus normal cells. Bak may, therefore, be a useful biomarker for the chemopreventive and therapeutic effects of perillyl alcohol.

The antitumor agent perillyl alcohol has chemotherapeutic activity toward pancreatic (1) and mammary cancers (2), and has promotion-phase chemopreventive activity against liver (3) and colon (4) cancers. Perillyl alcohol is more potent than d-limonene, a related monoterpene that also has widespread antitumor activity with low toxicity (5,6). Both perillyl alcohol and limonene are presently in clinical trials to evaluate their therapeutic activities (7).

The chemopreventive activity of perillyl alcohol toward liver (3) and colon (4) tumors is associated with an increase in apoptosis in the treated tumor cells. In apoptotic cells, the ‘death signal’ is executed through proteases and through endonucleases which cleave DNA at internucleosomal sites (8,9). Bcl-2 and closely related proteins form hetero- or homodimers with one another, and act upstream of these apoptotic proteases and endonucleases to modulate apoptosis in either a positive or negative manner (10–18). Whether a cell is protected from or induced to undergo apoptosis depends on the relative expression of Bcl-2-related suppressors or promoters of apoptosis. Bcl-2-related suppressors of apoptosis include Bcl-2 (10,11), Bcl-XL (17,18), Bag-1, and Mcl-1, while Bcl-2-related promoters of apoptosis include Bax (10,11), Bak (12–15), Bcl-XL (18), Bad and Nbk (9). The effects of perillyl alcohol on these molecular regulators of apoptosis are presently unknown. In an effort to address the mechanisms responsible for the relative effects of perillyl alcohol on malignant versus non-malignant pancreatic cells, we tested the hypotheses that perillyl alcohol would induce apoptosis and modulate the expression of Bcl-2 related proteins in pancreatic tumor cells, but would have little or no effect on non-malignant cells.

Immortalized, non-malignant D27 hamster pancreatic ductal epithelial cells were cultured on gelled collagen as described (19). B12/13 pancreatic ductal adenocarcinoma cells are derived from the D27 cells (20) and were cultured in the same medium. MIA PaCa-2, BxPC-3 and Panc-1 human pancreatic ductal adenocarcinoma cells were obtained from the American Type Culture Collection. MIA PaCa-2 and Panc-1 cells were cultured in DMEM and BxPC-3 cells were grown in RPMI 1640 medium, each supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 2 mM l-glutamine. Cell culture reagents were purchased from Life Technologies, and perillyl alcohol was purchased from Aldrich. Detection of [3H]thymidine incorporation into DNA was carried out as described (21). Apoptosis was measured by using Oncor’s ApopTag kit to detect free 3' ends of DNA (22). Protein content was determined by the method of Bradford (23) with reagents from BioRad. For Western blots, 40 µg of cell protein was analysed per well on a 10% SDS-polyacrylamide gel (24). Proteins were transferred to PVDF (BioRad) membrane (25) and the membrane was blocked, washed, incubated for 4 h in a 1/4000 dilution of anti-Bak, Bcl-2, Bcl-XL, Bcl-XS, or Bax antibody (Calbiochem), washed, incubated for 30 min in a 1/3000 dilution of anti-rabbit IgG/horseradish peroxidase conjugate (Amersham) and further washed. Chemiluminescent detection was carried out with reagents from Dupont. Quantitation of the Western blots was performed on a BioRad GS-670 imaging densitometer with Molecular Analyst 2.0 software (BioRad). Student’s t-test was carried out with StatisticaMac 4.1 software from StatSoft, Inc.

The studies described below addressed mechanisms by which the antitumor activity of perillyl alcohol toward pancreatic cancers is attained with low toxicity. As an in vitro model system for these studies, we utilized the immortalized, but non-malignant hamster pancreatic ductal epithelial cell line D27 and its fully malignant derivative cell line B12/13. We tested the hypothesis that perillyl alcohol would have differential effects on the growth of the malignant B12/13 cells versus the non-malignant D27 cells by measuring cell number at various timepoints in the absence and presence of the drug. While the cell growth rates of both the B12/13 and the D27 cells were suppressed by perillyl alcohol, the malignant B12/13 cells were more sensitive than the non-malignant D27 cells to the drug at every dose tested (Figure 1; P <0.05).

We then addressed whether these effects were due to changes in cell proliferation or apoptosis. First, the effects of perillyl alcohol on cell proliferation were assessed. Perillyl alcohol significantly inhibited [3H]thymidine incorporation per dish into the malignant B12/13 cells, but not into the non-malignant D27 cells (Table I), reflecting the differential effects of perillyl alcohol on cell growth rates described above (Figure 1).
DNA synthesis. We therefore hypothesized that the inhibitory effects of perillyl alcohol on the growth of malignant versus non-malignant pancreatic ductal epithelial cells. Malignant B12/13 and non-malignant D27 cells were plated in 35 mm dishes, 1000 cells per dish, and were grown for 48 h in the presence of 0, 100, 200, or 300 µM perillyl alcohol. The rate of cell proliferation was determined by subtracting the number of cells/dish (counted on a hemocytometer) on day 0 from the number of cells/dish on day 2. After plotting the cell growth rate as a percentage of the control versus perillyl alcohol concentration, the IC50 was determined by extrapolation. The data points (●, B12/13 cells; □, D27 cells) represent the mean ± SEM, n = 3. The results are representative of two independent experiments. At all drug concentrations, the D27 and the B12/13 values were significantly different (P < 0.05) by a two-tailed Student’s t-test. The IC50 values for perillyl alcohol were 150 µM for the B12/13 cells and 260 µM for the D27 cells.

Table I. Effects of perillyl alcohol on cell proliferation

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<th>Treatment</th>
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Cell proliferation was measured by detecting [3H]thymidine incorporation into DNA. B12/13 and D27 cells were treated with 0–300 µM perillyl alcohol and were then incubated for 0 or 60 min in medium containing 5 µCi/ml [methyl-3H]thymidine (48 Ci/mmol, Amersham). Cells were harvested and washed in phosphate-buffered saline (PBS). Cell pellets were resuspended in 0.1 µl PBS, frozen in liquid nitrogen, and stored at -80°C. The cell pellets were thawed at 4°C in an equal volume of 25% trichloroacetic acid, homogenized, incubated at 4°C for 10 min, and centrifuged at 12,000 rpm for 10 min. The pellet was washed twice with 12.5% trichloroacetic acid and once with cold acetone. The pellets were then air dried and dissolved in 100 µl of 0.1 M NaOH. One aliquot of this sample was analysed for protein content, and another aliquot was counted in a Beckman scintillation counter. The data were calculated by expressing cpm/well or cpm/µg protein as a percentage of the control value. The data represent the mean ± SEM, n = 4.

However, when these data were normalized by cell protein content (cpm/µg protein), thymidine incorporation into the perillyl alcohol-treated B12/13 or D27 cells did not differ from that of the controls (Table I), suggesting that perillyl alcohol was not simply suppressing cell proliferation by inhibiting DNA synthesis. We therefore hypothesized that the inhibitory effects of perillyl alcohol on B12/13 and D27 cell growth (Figure 1) were due to stimulation of apoptosis. B12/13 cells treated with 100, 300 and 500 µM perillyl alcohol, respectively, exhibited a 2.6 (P < 0.01 versus control), 8.8 (P < 0.01) and 18-fold (P < 0.01) higher rate of apoptosis than did untreated B12/13 cells (Figure 2). The non-malignant D27 cells treated with 300 or 500 µM perillyl alcohol exhibited a relatively small increase in the percentage of cells that were apoptotic (P < 0.01 versus control; Figure 2), and these effects were significantly less than those found in the malignant B12/13 cells (P < 0.005 for B12/13 versus D27 at 300 and 500 µM perillyl alcohol). We also tested the ability of perillyl alcohol to induce apoptosis in the human pancreatic ductal adenocarcinoma cell lines BxPC3, Panc1 and MIA PaCa2. The IC50 values for perillyl alcohol inhibition of cell growth were 200, 215 and 215 µM, for the BxPC3, Panc1 and MIA PaCa2 cells, respectively. Each human pancreatic carcinoma cell line was treated with 300 µM perillyl alcohol for 48 h and apoptosis assays were carried out as described above. Perillyl alcohol increased the percentage of cells undergoing apoptosis over that of untreated controls 5.7-fold in the BxPC3 cells (P < 0.01 versus control), 29-fold in the Panc-1 cells (P < 0.01) and 6.0-fold in the MIA PaCa2 cells (P < 0.05; Figure 3).

To investigate the mechanism by which perillyl alcohol induces apoptosis in pancreatic adenocarcinoma cells, we tested the hypothesis that perillyl alcohol would modulate the expression of Bcl-2 related proteins. The apoptosis-suppressing proteins Bcl-2 and Bcl-XL were undetectable by Western blot in both control and perillyl alcohol treated B12/13 and D27
three independent experiments. Cancer cell lines tested in this experiment contain mutant and wild-type p53 (20,28), arguing that the perillyl alcohol-induced increase in the expression of Bax, a protein of the Bcl-2 family that, like Bak and Bcl-X S , functions to increase apoptosis (12–15) and that Bak expression is high in intestinal epithelial cells that are undergoing apoptosis (26) are supportive of this hypothesis. Future experiments will address whether Bak is necessary and sufficient for the induction of apoptosis by perillyl alcohol in pancreatic adenocarcinoma cells.

The mechanism by which perillyl alcohol induces Bak expression is unknown at the present time. Bax, a protein of the Bcl-2 family that, like Bak and Bcl-X S , functions to stimulate apoptosis (10,16), is known to be induced by wild-type p53 in response to DNA damage (27). All of the pancreatic cancer cell lines tested in this experiment contain mutant and not wild-type p53 (10,16), and with the lack of effect of perillyl alcohol on DNA synthesis per µg cell protein in either cell line (Table I). The available data do not allow for quantitation to determine whether the magnitude of the apoptotic response by perillyl alcohol could account for all of the growth inhibition caused by the drug, since the TUNEL apoptosis assay detects only cells that have cleaved 3’ ends of DNA, and therefore underestimates all of the cells that have been committed to undergoing apoptosis. Taken together, the data presented here indicate that the antitumor activity of perillyl alcohol toward pancreatic tumors and its low toxicity to normal cells are likely due to selective stimulation of apoptosis in malignant versus non-malignant cells.

The Bcl-2 family of proteins are known to regulate apoptosis (9–18). Thus, we hypothesized that the induction of apoptosis by perillyl alcohol may be a result of a reduction in the expression of Bcl-2 related cell death suppressors or to an increase in the expression of Bcl-2 related cell death promoters. Perillyl alcohol clearly induced the expression of the apoptosis promoter Bak (Figures 4 and 5) in all pancreatic adenocarcinoma cell lines tested. Furthermore, the perillyl alcohol-induced increase in the expression of the proapoptotic protein Bak in the B12/13, but not in the D27 cells paralleled the preferential induction of apoptosis by the drug in the malignant B12/13 over the non-malignant D27 cells.

Together, these data point toward a common molecular mechanism for the antitumor activity of perillyl alcohol. It is likely that the induction of Bak by perillyl alcohol is responsible in part for the perillyl-alcohol induced apoptosis in pancreatic tumor cells. The observations that ectopic expression of Bak increases apoptosis (12–15) and that Bak expression is high in intestinal epithelial cells that are undergoing apoptosis (26) are supportive of this hypothesis. Future experiments will address whether Bak is necessary and sufficient for the induction of apoptosis by perillyl alcohol in pancreatic adenocarcinoma cells.

In the D27-B12/13 cell culture model system, we were able to investigate potential mechanisms by which the selective inhibition of malignant over normal cell growth by perillyl alcohol is attained. In every pancreatic tumor cell line tested, perillyl alcohol caused a marked increase in programeled death. Furthermore, the induction of apoptosis by perillyl alcohol was significantly greater in the malignant B12/13 cells ductal adenocarcinoma than in their non-malignant parental D27 ductal epithelial cells at every dose tested. These data are consistent with the differential sensitivity of these two cell lines to cell growth inhibition by perillyl alcohol (Figure 1) and with the lack of effect of perillyl alcohol on DNA synthesis per µg cell protein in either cell line (Table I). The available data do not allow for quantitation to determine whether the magnitude of the apoptotic response by perillyl alcohol could account for all of the growth inhibition caused by the drug, since the TUNEL apoptosis assay detects only cells that have cleaved 3’ ends of DNA, and therefore underestimates all of the cells that have been committed to undergoing apoptosis. Taken together, the data presented here indicate that the antitumor activity of perillyl alcohol toward pancreatic tumors and its low toxicity to normal cells are likely due to selective stimulation of apoptosis in malignant versus non-malignant cells.

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sugesting that an induction in Bak expression might also occur in other tumor types treated with perillyl alcohol.

Acknowledgements

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References