Cancer Therapy: Preclinical

PARP-1 Regulates Resistance of Pancreatic Cancer to TRAIL Therapy

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Abstract

Purpose: Activating extrinsic apoptotic pathways targeting death receptors (DR) using agonistic antibodies or TNF-related apoptosis-inducing ligand (TRAIL) is promising for cancer therapy. However, most pancreatic cancers are resistant to TRAIL therapy. The present studies aimed to identify combination therapies that enhance the efficacy of TRAIL therapy and to investigate the underlying mechanisms.

Experimental Design: A xenograft model in nude mice was used to determine pancreatic cancer tumorigenesis and therapeutic efficacy of TRA-8, a monoclonal agonistic antibody for DR5. Pancreatic cancer cells were used to characterize mechanisms underlying PARP-1 regulation of TRA-8–induced apoptosis in vitro.

Results: PARP-1 was found highly expressed in the TRA-8–resistant PANC-1 and Suit-2 cells, compared with TRA-8–sensitive BxPc-3 and MiaPaca-2. Inhibition of PARP-1 with a pharmacologic inhibitor sensitized PANC-1 and Suit2 cells to TRA-8–induced apoptosis in a dose-dependent manner. Furthermore, siRNAs specifically knocking down PARP-1 markedly enhanced TRA-8–induced apoptosis in vitro and augmented the efficacy of TRA-8 therapy on tumorigenesis in vivo. PARP-1 knockdown increased TRA-8–induced activation of caspase-8 in the death-induced signaling complex (DISC). Immunoprecipitation with DR5 antibody identified the recruitment of PARP-1 and PARP-1–mediated protein poly-ADP-ribosylation (pADPr) modification in the DR5-associated DISC. Further characterization revealed that PARP-1–mediated pADPr modification of caspase-8 inhibited caspase-8 activation, which may contribute to its function in regulating TRA-8 resistance.

Conclusions: Our studies provide molecular insights into a novel function of PARP-1 in regulating the extrinsic apoptosis machinery and also support interventions combining PARP-1 inhibitors with DR agonists for pancreatic cancer therapy. Clin Cancer Res; 19(17); 4750–9. ©2013 AACR.

Introduction

Pancreatic cancer is an important therapeutic challenge with nearly similar incidence and mortality rates. It is the most lethal cancer of the digestive system with a 5-year survival rate of 5% (1). Because of the resistance of pancreatic cancer to chemotherapy and radiotherapy, current treatments for pancreatic cancer are generally not curative with modest benefit (2, 3). We and others have shown that modulating death receptor (DR)-activated extrinsic apoptosis pathways may provide potential novel avenues for cancer therapy (4, 5). Upon ligand stimulation, DRs (Fas or death receptor 4/5, DR4/5) recruit Fas-associated death domain (FADD) and the initiator caspases, caspase-8 or caspase-10, resulting in formation of the death-inducing signaling complex (DISC). The recruited caspase-8 or caspase-10 undergoes autocatalytic cleavage and activation to trigger the caspase cascade that ultimately leads to apoptosis (4, 5).

Activating the DR4/5-mediated extrinsic apoptotic signaling pathway by TNF-related apoptosis-induced ligand (TRAIL) induces apoptosis of a variety of cancer cells including melanoma, colon, lung, breast, kidney, and pancreatic cancers (11, 12). With virtually no toxicity for normal cells, recombinant TRAIL or agonistic antibodies specifically targeting DR4/5 are currently being tested in several phase I–III clinical trials (12–14). Although TRAIL is a promising anticancer agent, many cancer cells, including pancreatic cancer cells, develop resistance to TRAIL-induced apoptosis. The resistance of cancer cells to TRAIL-induced apoptosis has been associated with mutations of DR5 receptor (15), increased expression of the decoy DRs DcR1 or DcR2 (16, 17), decreased expression of apoptotic protein...
transcaspase-8 (18), and increased expression of a number of antiapoptotic proteins, such as Bcl-2, Bcl-XL (19, 20) and the FLICE-like inhibitory protein (FLIP; refs. 21, 22).

Combination therapy with other anticancer agents has been shown to enhance the efficacy of TRAIL treatment for the resistant cancers (23–25). We and others have found that activating intrinsic apoptotic signaling pathways using gemcitabine (26), doxorubicin (27), or ionizing radiation (28) sensitizes resistant cancer cells to DR-induced apoptosis. These intrinsic apoptotic activators function via inducing mitochondria leakage or DNA damage (26–28).

The PARPs are key to repairing damaged DNA by modifying proteins associated with DNA repair (29). Inhibition of PARPs results in accumulation of irreparable damaged DNA and facilitates triggering of the intrinsic apoptotic signaling pathway in cancer therapy (29). PARP-1 is the most abundant of PARPs, accounting for 75% to 90% of the PARPs (29). Inhibition of PARP-1 through siRNA knockdown or chemical inhibitors for PARP has been used to treat cancers with DNA repair deficiencies, such as BRCA-deficient breast and ovarian cancer (30) as well as BRCA2-associated pancreatic cancer (31). In addition, combined use of PARP inhibitors with DNA-damaging reagents or radiation sensitizes glioma, ovarian, and pancreatic cancers to therapy (32–34).

The role of PARP-1 in DR-mediated extrinsic apoptotic signaling pathways is unknown. The present study provides the first evidence that PARP-1 inhibition sensitizes resistant pancreatic cancer cells to DR5-activated apoptosis and enhances the efficacy of DR5 antibody therapy for pancreatic cancers in a mouse tumor xenograft model. Furthermore, we have uncovered a novel mechanism of PARP-1 in regulating DR5-induced DISC recruitment and subsequent caspase-8 activation, which is independent of its known function in DNA repair. Our studies provide novel molecular insights into the function PARP-1 in the DR-activated apoptosis machinery and support the use of combined therapy with DR5 agonists and PARP inhibitors for resistant pancreatic tumors.

Materials and Methods

Cell culture, antibodies, and reagents

The human pancreatic cancer cell lines BxPc-3, MiaPaCa-2, PANC-1, and Suit-2 were from the American Type Culture Collection. BxPc-3 and Suit-2 cells were grown in RPMI-1640; MiaPaCa-2 and PANC-1 cells were grown in Dulbecco’s Modified Eagle’s Media (DMEM; Invitrogen) supplemented with penicillin (5 units/mL), streptomycin (5 μg/mL), and 10% heat-inactivated FBS. DR5 agonist antibody TRA-8 was generated as previously described (35). All antibodies used were commercially available, including FADD (Millipore), caspase-3 and pADPrt (Enzo Life), caspase-8 (BD Bioscience and Cell Signaling Technology), PARP-1 and GAPDH (Santa Cruz Biotech), and DR5 (Prosci).

Protein G agarose was from Invitrogen. Recombinant human PARP-1 protein was purchased from Enzo. β-NAD was from Sigma-Aldrich. Active human recombinant caspase-8 was from Research & Diagnostics. Full-length mouse recombinant caspase-8 was from Abcam.

Gene knockdown with lentivirus-delivered short hairpin RNA

Lentiviral constructs expressing a 21-nucleotide short hairpin RNA (shRNA) targeting human PARP-1 gene (GenBank accession number NM_003824.3) or human FADD gene (GenBank accession number NM_001618.3) were purchased from Open Biosystems. The constructs were packaged into lentivirus-like particles pseudotyped with the vesicular stomatitis virus glycoprotein as we previously described (35). Transduction was conducted by incubating cancer cells with lentivirus, and stably transduced cells were selected with puromycin (2 μg/mL).

Assessment of apoptosis

Apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (BD Biosciences) and analyzed by flow cytometry (BD Biosciences).

Western blot analysis

Proteins were extracted, quantified with a BCA protein assay kit (Thermo Scientific), separated by SDS-PAGE, and transferred to Immobilon P membranes (Millipore) as described previously (10). Membranes were blocked in 5% non-fat milk and incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. Signals were detected using the Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate Detection Kit (Millipore).
Immunoprecipitation

Five hundred micrograms of extracted proteins was incubated with 1 μg of antibodies as indicated in figure legends for 1 hour and subsequently incubated with 50 μL of 1:1 slurry of protein G-agarose beads overnight at 4°C. Beads were then washed, and 20 μL 2× Laemmli sample buffer was added to the beads followed by heating at 95°C for 5 minutes and chilling on ice. After brief centrifugation, proteins in the supernatant were analyzed by Western blotting with specific antibodies.

In vitro PADPr modification and caspase-8 activation assays

Recombinant full-length mouse Caspase-8 (300 ng, Abcam) was immunoprecipitated using antibody for caspase-8. Immunoprecipitated beads were incubated in 100 μL reaction buffer [100 mmol/L Tris-HCl (pH 8), 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol] with or without 100 ng of reaction buffer [100 mmol/L Tris-HCl (pH 8), 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol] with or without 100 ng of PARP-1 and 500 μmol/L β-NAD for 30 minutes at 37°C and then washed 3 times.

For caspase-8 activation analysis, immunoprecipitated beads were incubated in 100 μL reaction buffer [20 mmol/L HEPES (pH 7.5), 5 mmol/L dithiothreitol] with or without 100 ng of recombinant active human caspase-8 (Research & Diagnostic) at 37°C for 60 minutes. Beads were then washed and 20 μL 2× Laemmli sample buffer was added to the beads followed by heating at 95°C for 5 minutes and chilling on ice. After brief centrifugation, proteins in the supernatant were analyzed by Western blotting with specific antibodies.

Mouse xenograft model

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (Birmingham, AL). Male athymic nu/nu mice (6 weeks old, NCI-Frederick) were used for tumor inoculation. Briefly, control PANC-1 pancreatic cancer cells or Panc-1 cells with PARP-1 knockdown were inoculated subcutaneously into the flanks of mice (2×10⁶ cells in 200 mL PBS/site). Three weeks following tumor inoculation, mice were divided into two groups (8 mice per group), mice in the control group were injected with 0.9% sodium chloride, whereas Panc-1 and Suit-2 cells are resistant to TRA-8 (Fig. 1A). To understand the underlying mechanisms, we analyzed the expression of the receptor DR5, as well as the expression of an antiapoptotic protein FLIP (Fig. 1B) that has been shown to inhibit DR5-induced apoptosis (36). The expression of DR5 is higher in MiaPaCa-2 and Suit2 cells compared with that in BxPc-3 and Panc-1 cells, which was not correlated with their sensitivity to TRA-8-induced apoptosis. Similarly, the expression level of FLIP was not correlated to the resistance of the cells to TRA-8–induced apoptosis, suggesting that additional mechanisms may contribute to TRA-8 resistance.

We found that the expression of PARP-1 was correlated with TRA-8 resistance of the pancreatic cancer cells. The expression of PARP-1 was markedly higher in the TRA-8–resistant cell Panc-1 and Suit-2 cells, compared with that in the TRA-8–sensitive BxPc-3 and MiaPaCa-2 cells (Fig. 1B), suggesting a role of PARP-1 in TRA-8 resistance. Using a pharmacologic inhibitor for PARPs, PJ-34, we found that inhibition of PARP activity sensitizes resistant pancreatic cancer cells to TRA-8.

Knockdown of PARP-1 sensitizes TRAIL-resistant pancreatic cancer cells to TRA-8

The specific role of PARP-1 in mediating TRA-8 resistance of pancreatic cancer cells was further determined using shRNAs specific for PARP-1. Two lines of Panc-1 cells with PARP-1 knockdown were generated using shRNAs specifically targeting different regions of the PARP-1 gene. Knockdown of PARP-1 by the 2 shRNAs was confirmed by Western blot analysis (Fig. 2A, inserts). Knockdown of PARP-1 per se did not affect cell viability (Fig. 2A, white bars) but significantly increased the sensitivity of Panc-1 cells to TRA-8–induced apoptosis (Fig. 2A, black bars).
Similar to the observations with PJ-34 (Fig. 1D), PARP-1 knockdown increased TRA-8–induced activation of caspase-8 and caspase-3 (Fig. 2B).

**PARP-1 knockdown enhances efficacy of TRA-8 therapy in vivo**

To determine the effects of PARP-1 knockdown on the efficacy of TRA-8 therapy on tumorigenesis in vivo, we characterized tumorigenesis and response to TRA-8 treatment of PARP-1 knockdown PANC-1 cells in a mouse xenograft model. PANC-1 cells with scrambled shRNAs were used as controls. In the control groups, TRA-8 treatment did not reduce tumor size, further showing resistance of these cancer cells to TRA-8 therapy (Fig. 3A). In contrast, TRA-8 treatment effectively reduced tumor size of the PARP-1–knockdown cells (Fig. 3A). Western blot analysis determined that the expression of PARP-1 was maintained at a lower level in xenograft tumors of PARP-1–knockdown cells, which was not affected by TRA-8 treatment (Fig. 3B). Activation of caspase-8 and its downstream apoptosis effector caspase-3, indicated by cleaved caspase-8 and caspase-3, was shown in PARP-1–knockdown tumors from the TRA-8–treated mice (Fig. 3B). Furthermore, TUNEL staining of tumor sections showed significant increases in cell death in the PARP-1–knockdown tumors from TRA-8–treated mice (Fig. 3C).

**Activation of caspase-8 is responsible for PARP-1 knockdown–rendered TRA-8 sensitivity**

Because inhibition of PARP-1, by PJ-34 (Fig. 1) or by shRNAs (Fig. 2), increased TRA-8–induced activation of the upstream apoptosis initiator, caspase-8, we determined whether inhibition of caspase-8 may block the effects of PARP-1 inhibition. Z-IETD-FMK, a caspase-8 inhibitor, blocked TRA-8–induced apoptosis in the control PANC-1 cells. Moreover, inhibition of caspase-8 significantly reduced TRA-8–induced apoptosis in the PARP-1–knockdown cells (Fig. 4A). Consistently, caspase-8 inhibition resulted in
marked decreases in TRA-8–induced cleavage/activation of caspase-8 and its downstream caspase-3 in the PARP-1–knockdown cells (Fig. 4B).

Furthermore, knockdown of PARP-1 did not affect the expression of the death receptor DR5, the adaptor protein FADD, or the procaspase-8 (Fig. 4C). As procaspase-8 is

Figure 2. PARP-1 knockdown sensitizes TRAIL-resistant pancreatic cancer cells to TRA-8. A, apoptosis of control (shScr) or PARP-1 knockdown cells (PARP-1 KD) after exposure to TRA-8 (0.5 μg/mL) for 24 hours (n = 3 for each group; **, P < 0.001). Western blot analysis of the expression of PARP-1 in the control shScr cells and 2 lines of PARP-1 KD cells is shown in the inserts. B, Western blot analysis of the expression of caspase-8, caspase-3, and GAPDH in control shScr and 2 PARP-1 KD cells exposed to control (−) or TRA-8 (+, 0.5 μg/mL) for 8 hours. Representative blots from 3 independent experiments are shown.

Figure 3. PARP-1 knockdown enhances the efficacy of TRA-8 on tumorigenesis in mice. A, tumorigenesis of shScr cells and PARP-1 KD cells in nude mouse model as described in Materials and Methods. Tumor volumes and representative tumors in each group at 6 weeks after treatment are shown. NS, no significance; n = 8; **, P < 0.001. B, Western blot analysis of the expression of PARP-1, caspase-8, caspase-3, and GAPDH in isolated tumors. C, cell death analyzed by TUNEL staining in tumors; and quantitative analysis of TUNEL-positive cells as percentage of total cells in the tumor sections (n = 5 tumors in each group; **, P < 0.001 — bar, 50 μm).
known to be proteolytically cleaved and activated in the DR5-induced DISC, we analyzed DR5-recruited DISC proteins, including FADD and caspase-8, in response to TRA-8 stimulation. PARP-1 knockdown did not affect the recruitment of FADD into DR5-associated DISC in response to TRA-8 stimulation (Fig. 4C). As expected, TRA-8 induced the recruitment and activation of caspase-8, PARP-1, and DR5 in cell lysates, and Western blot analysis of DR5-associated DISC recruitment of FADD, caspase-8, and PARP-1. Representative blots from 3 independent experiments are shown.

Activation of DR5 induces pADPr modification of DISC component proteins

A key function of PARP-1 is to modify proteins by adding poly(ADP-ribose) (pADPr) chain to target proteins. Accordingly, we analyzed pADPr modification profile of the DR5-associated DISC proteins. Using anti-pADPr antibody, we determined that knockdown of PARP-1 significantly reduced overall protein pADPr modification in the PANC-1 cells (Fig. 5A, shScr). Furthermore, PARP-1 knockdown decreased TRA-8-induced pADPr modification on DR5, FADD, and caspase-8 (Fig. 5A, PARP-1 KD).

To further determine that pADPr modification of DR5, FADD, and caspase-8 occurred in the DR5-associated DISC, we knocked down FADD, the adaptor protein of DISC formation, with shRNA specifically targeting FADD (Fig. 5B). Knockdown of FADD did not affect the overall protein pADPr modification profile but decreased TRA-8-induced pADPr modification on DR5 and caspase-8 (Fig. 5B, pADPr), indicating that TRA-8-induced pADPr modification on DR5 and caspase-8 is dependent on FADD-mediated DISC formation (Fig. 5B).

pADPr modification inhibits caspase-8 activation

Using purified recombinant PARP-1 and caspase-8 proteins, a direct effect of PARP-1 on pADPr modification of caspase-8 was shown (Fig. 6A). Furthermore, the effect of pADPr modification of caspase-8 on its activation was characterized. In the absence of PARP-1, active caspase-8 cleaved procaspase-8 into cleaved p10/p18 fragments (Fig. 6B). However, decreased caspase-8 cleavage was observed when PARP-1 was added (Fig. 6B, last lane), indicating that pADPr modification of caspase-8 inhibited caspase-8 activation.

Discussion

TRAIL therapies have shown low toxicity in patients in several clinical trials (12–14). However, therapeutic effects...
protein pADPr modification, PARP-1 is an important regulator of the DNA base excision repair pathway and is essential for the maintenance of genomic integrity and cell survival in response to genotoxic insults (37, 38). PARP-1 elevation is associated with adverse outcomes of breast and ovarian cancers (39, 40). Consistently, we found that the expression of PARP-1 was increased in 2 TRA-8–resistant pancreatic cell lines, PANC-1 and Suit-2 cells, and inhibition of PARP-1 rendered the resistant cells more sensitive to TRA-8–induced apoptosis, showing the engagement of PARP-1 in TRA-8–resistant pancreatic cancer cells.

Consistent with its essential role in DNA repair, PARP-1 antagonists, used as a monotherapy for tumors with DNA repair deficiencies or in combination with DNA damage–inducing agents, increase accumulation of damaged DNA that triggers intrinsic apoptotic pathways (31–34). In pancreatic cancer, PARP inhibitors enhance the effects of chemotherapy with DNA-alkylating agents or radiation therapy, both via promoting DNA damage–induced intrinsic apoptotic signaling pathways. The present studies have provided the first evidence that PARP-1 inhibition alone did not induce apoptosis of pancreatic cancer cells but enhanced TRA-8–induced apoptosis, suggesting that PARP-1 regulates TRA-8–induced extrinsic apoptosis via a mechanism independent of its function in DNA repair.

The expression of PARP-1 was identified in both nucleus and cytoplasm of the PANC-1 and Suit-2 cells (data not shown).
PARP-1 Regulates DR5-Mediated Apoptosis

In the context of pancreatic cancer, PARP-1 knockdown sensitized resistant cancer cells to TRA-8-induced apoptosis in vitro and enhanced the efficacy of TRA-8 therapy of pancreatic tumors in vivo. PARP-1 was identified in the DR5-associated complex, which induced PARP modification of caspase-8 in the TRA-8-activated DISC and modulated caspase-8 activation. Therefore, inhibition of PARP-1 blocks PARP modification of caspase-8, which facilitates DR5-mediated activation of caspase-8 in the DISC, and thus sensitizing resistant pancreatic cancer cells to TRA-8-induced apoptosis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): K. Yuan, Y. Sun, J.M. McDonald, Y. Chen
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