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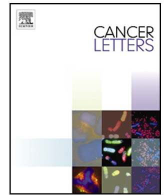

Original Articles

Smac mimetic triggers necroptosis in pancreatic carcinoma cells when caspase activation is blocked

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ABSTRACT

Evasion of apoptosis represents a key mechanism of treatment resistance of pancreatic cancer (PC) and contributes to the poor prognosis of this cancer type. Here, we report that induction of necroptosis is an alternative strategy to trigger programmed cell death in apoptosis-resistant PC cells. We show that the second mitochondrial activator of caspases (Smac) mimetic BV6 that antagonizes inhibitor of apoptosis (IAP) proteins induces necroptosis in PC cells in which apoptosis is blocked by the caspase inhibitor zVAD.fmk. Intriguingly, BV6 switches autocrine/paracrine production of tumor necrosis factor (TNF)α by PC cells into a death signal and also acts in concert with exogenously supplied TNFα to trigger necroptosis, when caspase activation is simultaneously blocked. BV6 stimulates TNFα production and formation of the receptor-interacting protein (RIP)1/RIP3-containing necrosome complex in PC cells. Knockdown of TNF receptor 1 (TNFR1) protects PC cells from BV6- or BV6/TNFα-mediated cell death, demonstrating that TNFα autocrine/paracrine signaling by PC cells contributes to BV6-induced necroptosis. Importantly, genetic silencing of receptor interacting protein kinase 3 (RIPK3) or mixed lineage kinase domain-like protein (MLKL) significantly rescues PC cells from BV6- or BV6/TNFα-induced cell death. Similarly, pharmacological inhibition of RIP1, RIP3 or MLKL significantly reduces BV6- or BV6/TNFα-stimulated cell death. By demonstrating that Smac mimetics can bypass resistance to apoptosis by triggering necroptosis as an alternative form of programmed cell death, our findings have important implications for the design of new treatment concepts for PC.

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Introduction

Pancreatic cancer (PC) is currently the fourth most common cause of death in cancer patients and PC-related death is expected to become the second leading cause of cancer deaths in the near future [1–3]. Patients who suffer from PC have the lowest survival rate among all patients with malignant diseases [4]. This highlights the high medical need to develop novel treatment strategies.

Evasion of programmed cell death such as apoptosis represents a key mechanism of treatment resistance of PC [5]. Besides apoptosis, necroptosis represents a newly discovered pathway of programmed cell death that is induced, for example, by death receptor ligands such as TNFα [6]. TNFα-stimulated necroptosis is initiated by binding of TNFα to its cognate receptor TNFR1 on the cell surface, which leads to the formation of the necrosome, a signaling complex that contains RIP1 and RIP3 as its core components [7]. RIP3 then phosphorylates and activates the pseudokinase MLKL, which promotes oligomerization of MLKL and its translocation to the plasma membrane [8,9]. MLKL promotes necroptotic cell death by disrupting the integrity of the plasma membrane via pore formation [8,9]. Apoptosis and necroptosis pathways are intertwined; for example, caspase-8-mediated proteolytic processing of RIP1 has been reported to shut off necroptosis [10].

The protein family of inhibitors of apoptosis (IAP) proteins is well known to inhibit cell death [11]. X-linked IAP (XIAP) blocks apoptosis by antagonizing caspase activation and cellular IAP (cIAP) proteins promote K63-linked polyubiquitination of RIP1, resulting in Nuclear Factor kappaB (NF-κB) activation and inhibition of cell death [11]. High expression levels of XIAP have been identified as a mechanism contributing to the resistance of PC cells to apoptosis [12,13]. Small-molecule inhibitors that neutralize XIAP, cIAP1 and

Abbreviations: cIAP, cellular IAP; FADD, Fas-associated protein with death domain; FCS, fetal calf serum; GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase; IAP, inhibitor of apoptosis; MLKL, mixed lineage kinase domain-like protein; Nec-1, Necrostatin-1; NF-κB, Nuclear Factor kappaB; NSA, necrosulfonamide; PC, pancreatic cancer; PI, propidium iodide; RIP, receptor-interacting protein; RIPK, receptor interacting protein kinase; Smac, second mitochondrial activator of caspases; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAIL, tumor-necrosis-factor-related apoptosis-inducing ligand; XIAP, X-linked IAP.

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cIAP2 such as Smac mimetics are considered as a promising approach to reactivate programmed cell death in cancer and are currently being evaluated in early clinical trials [14]. In preclinical models of PC, Smac mimetics have been shown to sensitize cells to death receptor-, chemotherapy- or irradiation-induced apoptosis [15–19]. For apoptosis-resistant types of cancer, induction of necroptosis is considered as an alternative strategy to elicit cancer cell death [20,21]. In the present study, we therefore investigated the question as to whether or not the bivalent Smac mimetic BV6 can bypass apoptosis resistance of PC cells by engaging necroptosis.

Materials and methods

Cell culture and chemicals

PC cell lines (AsPc-1, BxPc-3, Capan-1, Capan-2, MiaPaCa-2, PaTu8902, PaTu8988T, SU.86.86., Panc-1, DanG) were kindly provided by D. Saur (Munich, Germany) or A. Piiper (Frankfurt, Germany) and were cultured in RPMI 1640 or DMEM (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% or 20% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mM Pyruvate (Invitrogen, Karlsruhe, Germany) and 1% penicillin–streptomycin (Invitrogen). The Smac mimetic BV6, which neutralizes XIAP, cIAP1 and cIAP2 [22], was kindly provided by Genentech, Inc. (South San Francisco, CA, USA). Caspase inhibitor zVAD.fmk was obtained from Bachem (Heidelberg, Germany), TNFα and Necrostatin-1 (Nec-1) from Biomol (Hamburg, Germany), necrosulfonamide (NSA) from Toronto Research Chemicals Inc. (North York, CA), GSK'872 from Merck (Darmstadt, Germany), and zVAD.fmk from R&D Systems (Wiesbaden, Germany). Enbrel was kindly provided by Pfizer (Berlin, Germany). All chemicals were purchased by Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany) unless indicated otherwise. Cells were preincubated with zVAD.fmk, Nec-1, NSA, GSK'872 or Enbrel for 1 hour before treatment with BV6.

Western blot analysis and immunoprecipitation

Western blot analysis was performed as described previously [23] using the following antibodies: rabbit anti-cIAP2 (Epitomics, Burlingame, CA, USA), goat anti-cIAP1 (R&D Systems, Wiesbaden, Germany), mouse anti-Fas-associated protein with death domain (FADD) and mouse anti-RIP1 (BD Biosciences, Heidelberg, Germany), rabbit anti-RIP3 (Novus Biologicals, Littleton, CO, USA), rabbit anti-MLKL (GeneTex, Irvine, CA, USA), mouse anti-caspase-8 (Enzo Life Science, Lörrach, Germany), mouse anti-β-actin (Sigma-Aldrich) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (HyTest, Turku, Finland). Secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced chemiluminescence were used for detection (Amersham Bioscience, Freiburg, Germany). Alternatively, secondary antibodies labeled with IRDye infrared dyes were used for fluorescence detection (Odyssey Imaging System, LI-COR Bioscience, Bad Homburg, Germany). All Western blots shown are representative of at least two independent experiments. Immunoprecipitation was performed as previously described [24] using RIPA buffer for cell lysis.

Determination of cell death

Cell death was determined by analysis using propidium iodide (PI) staining and flow cytometry (FACSCanto II, BD Biosciences) as described previously [25].

Quantitative RT-PCR

TNFα mRNA levels were determined by quantitative real-time PCR (qRT-PCR) analysis. Total RNA extraction and cDNA synthesis were performed as previously described [26]. TNFα mRNA levels were assessed by Taqman Gene Expression Assay purchased from Life Technologies and the levels of 28S rRNA by SYBR® Green qPCR assay from Applied Biosystems (Darmstadt, Germany) as previously described [27]. At least three independent experiments in duplicate were performed for each gene.

Gene silencing

For transient knockdown by small interfering RNA (siRNA) cells were reversely transfected for 48 hours (AsPc-1) or 72 hours (Capan-1) using 10 nM (AsPc-1) or 20 nM (Capan-1) Silencer® Select siRNAs against RIP1 (#1: s16651, #2: s16653), RIP3 (#1: s21740, #2: s21741), MLKL (#1: s47087, #2: s47088), TNFR1 (#1: s14266, #2: s14267), or the respective concentrations of non-targeting control siRNA (no. 4390843). 48 h (AsPc-1) or 72 h (Capan-1) after transfection, gene silencing was confirmed by Western blotting and cells were treated for determination of cell death.

Statistical analysis

Statistical significance, when comparing two groups, was assessed by Student's t-test (two-tailed distribution, two-sample with equal variance) using Microsoft Excel (Microsoft Deutschland GmbH, Unterschleißheim, Germany).

Results

PC cell lines express key components of necroptosis signaling

Initially, we determined in a panel of PC cell lines the expression levels of key components of necroptotic and apoptotic signaling, i.e. RIP1, RIP3, MLKL, caspase-8 and FADD. Jurkat leukemia cells were used as positive control. All tested PC cell lines exhibited protein expression of RIP1, MLKL, caspase-8 and FADD, although at different levels, while RIP3 protein was detected in 6/10 PC cell lines (Fig. 1A). We therefore selected AsPc-1, Capan-1, BxPc-3 and DanG cell lines for subsequent experiments to explore the regulation of necroptosis in PC, since they express RIP3 protein.

Since Smac mimetics such as BV6 have been described to trigger proteasomal degradation of cIAP proteins [22,28,29], we determined protein levels of cIAP1 and cIAP2 after treatment with BV6 to examine whether BV6 engages its known targets in PC cells. Indeed, BV6 caused a marked downregulation of cIAP1 and cIAP2 (Fig. 1B).

PC cells are susceptible to BV6- or BV6/TNFα-induced cell death upon caspase inhibition

To investigate the ability of PC cells to undergo necroptotic cell death, we treated PC cells with BV6 alone or in combination with

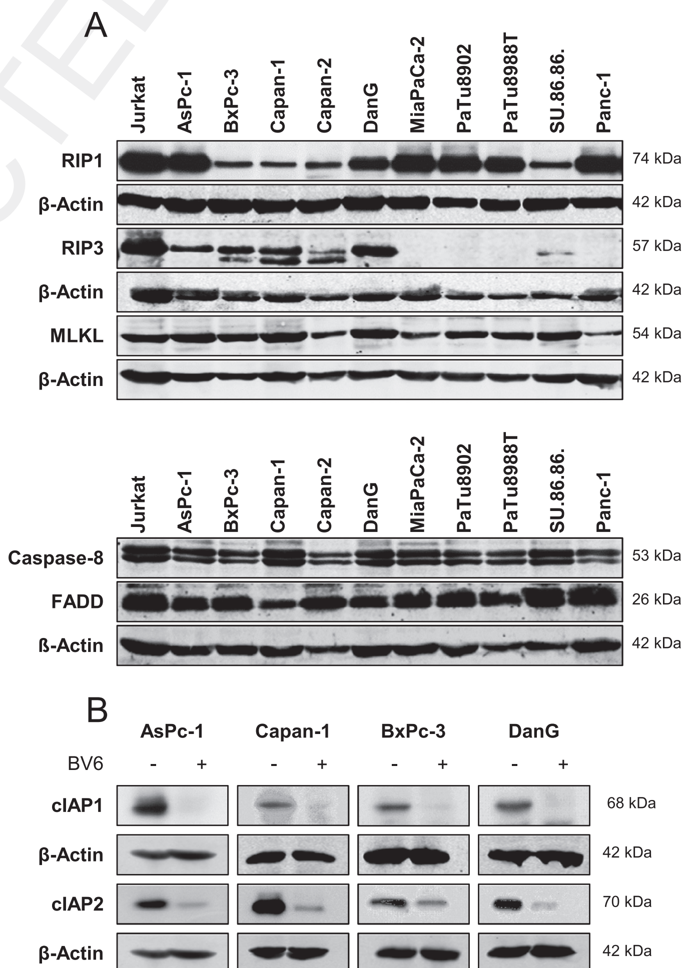

Fig. 1. PC cell lines express key components of necroptosis signaling. A, Protein expression of RIP1, RIP3, MLKL, caspase-8 and FADD was analyzed in PC cell lines by Western blotting, and Jurkat cells were used as positive control. B, AsPc-1, Capan-1, BxPc-3, DanG cells were treated with 5 μM BV6 for 6 hours. Protein expression of cIAP1 and cIAP2 was analyzed by Western blotting, and β-actin served as loading control.

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