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Apoptosis pathways and their therapeutic exploitation in pancreatic cancer

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Abstract

Resistance to apoptosis (programmed cell death) is a characteristic feature of human malignancies including pancreatic cancer, which is one of the leading causes of cancer deaths in the western world. Defects in this intrinsic cell death program can contribute to the multistep process of tumorigenesis, because too little cell death can disturb tissue homeostasis. Further, blockade of apoptosis pathways can cause treatment failure, because intact apoptosis signalling cascades largely mediate therapy-induced cytotoxicity. The elucidation of apoptosis pathways in pancreatic carcinoma over the last decade has resulted in the identification of various molecular defects. How apoptosis pathways can be exploited for the treatment of pancreatic cancer will be discussed in this review.

Keywords: apoptosis • pancreatic cancer • TRAIL • IAPs • mitochondria

Introduction

A characteristic feature of human cancers is a deregulation of the normal balance between proliferation and cell death, which is crucial to maintain tissue homeostasis [1]. Accordingly, too little cell death can contribute to tumour formation [2]. Apoptosis (programmed cell death) is a form of cell death that is highly conserved throughout evolution and plays an important role on the control of various physiological and pathological processes [3]. Evasion of apoptosis is one of the hallmarks of human cancers including pancreatic carcinoma. In addition, defects in apoptosis programs can contribute to the primary or acquired resistance of pancreatic carcinoma to therapies that are currently used in the clinic, because the response of cancer cells to current treatment approaches is, to a large extent, due to their ability to undergo cell death in response to cytotoxic stimuli [4–6]. Thus, a better understanding of the regulation of apoptosis signalling in pancreatic cancer cells is expected to identify novel molecular targets in pancreatic cancer that can be exploited for the development of molecular targeted therapies.

Pancreatic cancer

Pancreatic cancer is one of the leading causes of cancer deaths in the western world with steadily rising numbers [7, 8]. The prognosis of patients with the diagnosis of pancreatic cancer is very poor despite intensive protocols [7]. Resistance of pancreatic cancer to current treatment regimens contributes to the dismal prognosis of this malignancy and presents a major challenge in oncology [9]. Evasion of apoptosis, the cell's intrinsic cell death program, is a characteristic feature of human cancers including pancreatic carcinoma and promotes the development and progression of pancreatic cancer [10]. Also, resistance to apoptosis considerably contributes to treatment failure in pancreatic cancer, because most anticancer therapies including chemotherapy, radiation and immunotherapy primarily exert their antitumour effects by triggering apoptosis in cancer cells. Thus, current attempts to improve the survival of patients with pancreatic cancer will have to include strategies that target defective apoptosis programs.

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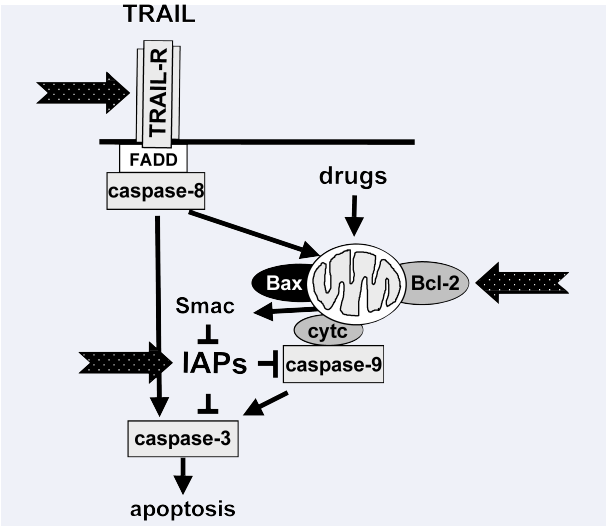

Fig. 1 Apoptosis pathways. Apoptosis pathways can be initiated by ligation of death receptors such as TRAIL receptors (TRAIL-Rs) by their respective ligands, *e.g.* TRAIL, followed by receptor trimerization, recruitment of adaptor molecules (FADD) and activation of caspase-8 (receptor pathway). The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c or Smac from mitochondria in the cytosol. Apoptosis can be inhibited by Bcl-2 or by 'inhibitor of apoptosis proteins' (IAPs). Smac promotes apoptosis by neutralizing IAP-mediated inhibition of caspase-3 and -9. See text for more details.

Apoptosis pathways

Apoptosis pathways can be triggered by a wide range of stimuli, both originating from the outside of the cell, *e.g.* death receptors or cytotoxic agents, or from intracellular signals, *e.g.* reactive oxygen species. In caspase-dependent apoptosis, activation of apoptosis pathways eventually leads to activation of caspases that function as common death effector molecules [11]. Caspases are cysteine proteases that are synthesized as inactive proenzymes and become activated upon cleavage [11].

The death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway are the two principal pathways of apoptosis that fuel into activation of caspases (Fig. 1) [3]. Ligation of death receptors of the tumour necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or agonistic TNF-related apoptosis-inducing ligand (TRAIL) receptors results in activation of the initiator caspase-8 [12]. Activated caspase-8 in turn can either directly propagate the apoptosis signal by cleavaging effector caspases such as caspase-3 or alternatively, may engage the mitochondrial pathway *via* the cleavage of Bid. The cleaved form of Bid (tBid) then translocates to mitochondria to cause mitochondrial perturbations, which lead to the release of apoptogenic factors from the mitochondrial intermembrane space into the cytosol [13, 14]. Such factors comprise cytochrome c, apoptosis inducing factor (AIF), Smac/direct IAP binding protein with low pI (DIABLO), Omi/HtrA2 or AIF. The release of cytochrome c triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Smac/DIABLO or Omi/HtrA2 promotes caspase activation through neutralizing the inhibitory effects of IAPs [14]. In addition, AIF has been described to mediate caspase-independent death and large scale DNA fragmentation after release from mitochondria [15]. Most cytotoxic drugs are considered to primarily initiate cell death by triggering a cytochrome c/Apaf-1/caspase-9 dependent pathway linked to mitochondria [16].

Besides apoptosis, non-apoptotic modes of cell death also exist, for example necrosis, autophagy, mitotic catastrophe, lysosomal cell death or paraptosis [17]. Non-caspase proteases such as calpains or cathepsins may be involved in these alternative forms of cell death [17]. It is increasingly becoming clear that the form of cell death is highly context related and may depend among other factors on the type, strength or duration of the stimulus as well as on the cell type.

Exploiting apoptosis pathways for pancreatic cancer therapy

Exploiting the death receptor pathway for pancreatic cancer therapy

Death receptors belong to the TNF receptor gene superfamily that all harbour an extracellular domain for binding of their corresponding ligands, a transmembrane part and an intracellular domain called 'death domain' [12, 18]. This death domain is crucial for transmitting the death signal from the cell's surface to intracellular signalling pathways and serves as a docking platform for the recruitment of adaptor and signalling molecules [12, 18]. CD95 (APO-1/Fas), TNF receptor 1 (TNFRI) and TRAIL receptors are the best-characterized death receptors and their corresponding ligands of the TNF superfamily are CD95 ligand, TNF-α and TRAIL. Binding of CD95 ligand or TRAIL to their corresponding receptors leads to the recruitment of the adaptor molecule Fas-associated death domain (FADD) and of caspase-8 to the activated receptor to form a multimeric complex at the plasma membrane, *i.e.* the death inducing signalling complex (DISC) [12, 19]. This in turn leads to caspase-8 activation, which can then directly cleave downstream effector caspases such as caspase-3 [12].

The CD95 receptor/CD95 ligand system is a key regulator of apoptosis in the immune system as well as in immunosurveillance of cancer [18]. CD95 is expressed on activated lymphocytes, on a variety of tissues of lymphoid origin and also on tumour cells [18]. CD95 ligand is produced by cytotoxic T cells and can trigger autocrine suicide or paracrine death in lymphocytes and also contributes to tumour immunosurveillance by killing cancer cells [18]. For example, pancreatic cancer cells have been reported to evade Fas-mediated immune surveillance by expressing a non-functional CD95 receptor or by aberrant expression of CD95 ligand on their surface to 'counterattack' cytotoxic T-cells [20].

The concept to exploit the death receptor system in human cancers for therapeutic purposes is attractive, because death receptor receptors can directly engage an inbuilt genetic program of cell death in cancer cells.

To this end, TRAIL is considered as the most promising clinical candidate among the death receptor ligands, because TRAIL has shown some selectivity for cancer over non-malignant cells [21, 22]. The molecular events that could explain the differential sensitivity of cancer *versus* normal cells for TRAIL have not exactly been identified despite intensive efforts [23]. TRAIL receptor system is complex, because five TRAIL receptors with distinct functions exist [21]. There are two agonistic TRAIL receptors, *i.e.* TRAIL-R1 and TRAIL-R2, which contain a conserved cytoplasmic death domain motif, and thus, can engage the cell's apoptotic machinery upon binding of TRAIL [21]. TRAIL-R3, TRAIL-R4 and TRAIL-R5 are antagonistic decoy receptors, which bind TRAIL, but do not transmit a death signal [21]. TRAIL-R3 is a glycosyl-phosphatidylinositol (GPI)-anchored cell surface protein and completely lacks the cytoplasmic tail with the death domain, whereas TRAIL-R4 has a truncated cytoplasmic death domain [21]. Besides these four membrane-associated TRAIL receptors, osteoprotegerin is a soluble decoy receptor that can bind TRAIL and is involved in regulation of osteoclastogenesis [21].

Analysis of TRAIL and its receptors in human pancreatic cancer samples indicate that at least one of the agonistic TRAIL receptors is usually expressed in pancreatic carcinoma tissue. To this end, TRAIL, TRAIL-R1 and TRAIL-R2 were described to be concomitantly expressed at mRNA and protein levels in pancreatic cancer tissue [24]. Similarly, analysis of mRNA levels showed expression of TRAIL and its four receptors other than osteoprotegerin in most cases of pancreatic ductal adenocarcinoma and also in normal pancreatic tissue, while osteoprotegerin expression was restricted to pancreatic cancer samples [25]. A recent immunohistochemical analysis showed increased TRAIL-R1 and TRAIL-R4 expression in pancreatic ductal adenocarcinoma compared with non-cancer patients [26]. By comparison, TRAIL-R3 mRNA and protein expression were detected at low levels in pancreatic cancers and normal pancreatic tissues [27]. TRAIL-R4 mRNA and protein were found at moderate to high levels in pancreatic cancer tissues, while weak or no expression was detected in the normal pancreas [27].

In addition, the majority of pancreatic carcinoma cell lines were reported to express the essential signalling molecules of the TRAIL system, including at least one of the agonistic TRAIL receptors and caspase-8 [28–30]. However, despite expression of TRAIL receptors, the majority of pancreatic cancer cell lines proved to be insensitive towards TRAIL-mediated apoptosis [30, 31]. Therefore, various combination therapies were developed to bypass the resistance of pancreatic carcinoma cells to TRAIL.

For example, camptothecin, cisplatin and celecoxib were found to enhance the sensitivity of pancreatic carcinoma cells to TRAIL by down-regulating c-FLIP expression [32]. Similarly, knockdown of c-FLIP by RNAi significantly enhanced TRAIL-induced apoptosis [32]. In addition, TRAIL in combination with gemcitabine was shown to result in a greater antitumour effect than either therapy used alone on patient pancreatic adenocarcinomas grown as xenografts in severe combined immunodeficiency disease (SCID) mice [33]. Similarly, combination administration of an anti-TRAIL receptor 2 antibody, *i.e.* TRA-8, together with CPT-11 resulted in tumour regression in an orthotopic model of pancreatic cancer [34]. Moreover, 2-Methoxyestradiol, a physiologic metabolite of 17β-estradiol, was reported to increase the response to TRAIL by up-regulating TRAIL receptors *via* generation of oxidative stress and JNK activation [35]. Further, inhibition of the cyclin-dependent kinase 4 (CDK4) by a pharmacological inhibitor or by RNAi markedly increased the sensitivity towards TRAIL [36]. Also, concomitant treatment with the proteasome inhibitor bortezomib significantly sensitized pancreatic carcinoma cells to TRAIL-induced apoptosis [37]. Analysis of molecular events revealed that bortezomib triggered up-regulation of TRAIL-R1/TRAIL-R2, formation of the TRAIL DISC, down-regulation of the anti-apoptotic protein c-FLIP$_L$ and accumulation of the pro-apoptotic protein Bak, thereby tipping the balance towards apoptosis [37]. Moreover, the addition of certain phenoxazine derivatives enhanced the cytotoxicity of TRAIL [38]. While there are additional anticancer agents that have been reported to act in concert with TRAIL in pancreatic cancer, the examples given above support the concept that TRAIL-based regimens of pancreatic cancer will likely be combination protocols.

In a search for biomarkers that predict sensitivity to TRAIL, a correlation between the expression of specific O-glycosylation enzymes and the response to TRAIL was recently identified in certain cancer types including pancreatic cancer [39]. O-glycosyltransferases are overexpressed in several cancers and may regulate TRAIL-induced apoptosis *via* modulation of TRAIL-R1 or -R2, as these two agonistic TRAIL receptors harbour O-glycosylation sites that facilitate TRAIL-mediated receptor aggregation, DISC formation and activation of caspase-8 [39].

In order to exploit the TRAIL system for clinical application, various agents have been developed in recent years [22]. These include recombinant soluble TRAIL as well as agonistic antibodies directed against the apoptosis inducing TRAIL receptors, *i.e.* TRAIL-R1 and -R2 [40, 41]. Apomab, an agonistic antibody against TRAIL-R2, was recently reported to show antitumour activity against pancreatic cancer as single agent and also in combination with gemcitabine [42]. As far as pancreatic carcinoma is concerned, fully human TRAIL-R1 monoclonal antibodies are currently under evaluation in a phase I clinical trial for advanced solid tumours in combination with gemcitabine and cisplatin [43] (Table 1).

It is important to note, TRAIL has been reported to exert also non-apoptotic functions under certain conditions, which has been linked to its ability to stimulate survival cascades such as NF-κB, PI3K/Akt or Ras/Raf/ERK pathways [44]. For example, TRAIL promoted metastasis of human pancreatic carcinoma in an orthotopic mouse model [45]. This indicates that the design of TRAIL-based combination regimens should take into consideration not only to enhance apoptosis induction by TRAIL, but in addition to prevent the tumour promoting activities of TRAIL.

Table 1 Examples of apoptosis targeted drugs in clinical trials

Name	Clinical trial	Cancer type	References
(1) TRAIL receptor agonists			
TRAIL	Phase I	Solid tumours, NHL	[40]
TRAIL-R1/2 mAb	Phase I	Solid tumours	[41, 43]
(2) IAP targeting agents			
XIAP antisense	Phase I/II	Solid tumours, AML	[54]
IAP inhibitors	Phase I	Solid tumours	[46]
(3) Bcl-2/Bcl-XL targeting agents			
Bcl-2 antisense	Phase I–III	Solid tumours, leukaemia/lymphoma	[76]
Bcl-2/Bcl-XL inhibitor	Phase I	Leukaemia	[72]

Targeting 'inhibitor of apoptosis proteins' (IAPs) for pancreatic cancer therapy

IAPs are a family of endogenous caspase inhibitors that comprise eight human analogues, *i.e.* XIAP, cIAP1, cIAP2, surviving, livin (ML-IAP), NAIP, Bruce (apollon) and ILP-2 [46]. Classification as IAP protein requires the existence of at least one baculovirus IAP repeat (BIR) domain, which is also the domain that interacts with caspases. Among the IAP family proteins, XIAP is considered to exert the most potent anti-apoptotic effects [47] and blocks apoptosis by binding and inhibiting active caspase-3 and -7 and by preventing caspase-9 activation [46].

Survivin is an IAP family protein that besides regulating apoptosis survivin also controls mitotic events [48]. IAPs are negatively regulated at several levels, *e.g.* by mitochondrial proteins such as Smac/DIABLO or Omi/HtrA2 or by nuclear proteins such as XIAP-associated factor 1. These endogenous IAP antagonists translocate into the cytosol upon induction of apoptosis and neutralize the anti-apoptotic function of IAPs through binding to IAPs [14]. In addition, IAPs are inactivated by caspase-mediated cleavage. Furthermore, expression levels of IAPs are tightly controlled by ubiquitination through the RING domain of IAPs *via* auto- and heteroubiquitination.

Several IAP proteins were reported to be expressed at high levels in pancreatic cancer compared to non-malignant pancreatic ductal cells or pancreatic tissue, *i.e.* XIAP, cIAP2, survivin and livin [49–51]. Expression and/or function of IAPs can be targeted in pancreatic cancer by several approaches. Because XIAP has been shown to posses the most potent anti-apoptotic properties among the IAP proteins, most strategies developed so far are directed against XIAP. For example, expression levels of XIAP can be down-regulated by RNA interference (RNAi) of antisense oligonucleotides. RNAi-mediated knockdown of XIAP was shown to significantly enhance apoptosis upon treatment with TRAIL, agonistic anti-CD95 antibodies or γ-irradiation and also to suppress clonogenic growth of pancreatic carcinoma cells [30, 52]. Importantly, XIAP inhibition in combination with TRAIL was shown to even break Bcl-2-imposed resistance by switching type II cells, which depend on the mitochondrial contribution to TRAIL-induced apoptosis, to type I cells in which TRAIL-mediated apoptosis proceeds irrespective of high Bcl-2 levels [53]. In a tumour regression model in xenograft-bearing mice, inhibition of XIAP cooperated with TRAIL to cause regression of established pancreatic carcinoma [53]. In some pancreatic cancer cell lines, down-regulation of XIAP or cIAP2 increased the response to anticancer drugs including doxorubicin, paclitaxel, gemcitabine and cisplatin, whereas the effect of 5-fluorouracil was only marginally affected [50, 51].

Moreover, XIAP antisense oligonucleotides were developed to antagonize XIAP that are currently evaluated in early clinical trials [54]. Loss of XIAP protein upon administration of XIAP antisense oligonucleotides correlated with increased sensitization to TRAIL-mediated apoptosis in a pancreatic carcinoma cell line [55]. The binding groove of the BIR3 domain of XIAP, to which Smac binds to once it is released from the mitochondrial interspace into the cytosol, has served in many drug discovery approaches as a target for the development of small molecule inhibitors of XIAP [56]. As far as pancreatic cancer is concerned, Smac peptides that consist of the N-terminal part of Smac that is essential for its binding to XIAP were found to sensitize pancreatic carcinoma cells to death-receptor ligation or cytotoxic drugs [57]. To facilitate intracellular delivery, Smac peptides were linked to a carrier such as the protein transduction motif of the HIV Tat protein [57]. Above all, Smac peptidomimetics were developed on the basis of the 3D structure of Smac in complex with XIAP BIR3 [58]. These Smac peptidomimetics were reported to enhance radiosensitivity of pancreatic carcinoma cells by promoting γ-irradiation-induced caspase activation and apoptosis [52].

Furthermore, the natural product embelin from the Japanese Ardisia herb was discovered in a computational screening of a traditional herbal medicine 3D structure database as a cell-permeable, non-peptidic, small-molecular weight inhibitor of XIAP [59]. In cell-based assays, embelin was shown to increase TRAIL-induced apoptosis in pancreatic carcinoma cells [60].

There are also small molecule compounds that were designed against the BIR2 domain of XIAP. For example, screening of a polyphenylurea library yielded several non-peptidic molecules that displayed potent binding to BIR2 of XIAP [61, 62]. Because XIAP interacts with effector caspase-3 and -7 *via* its BIR2 domain, while the BIR3 domain is responsible for its binding to the initiator caspase-9, it has been argued that targeting the BIR2 domain might be superior over BIR3 [61, 62]. Accordingly, these polyphenylurea compounds triggered apoptosis in pancreatic carcinoma cells without the requirement of an additional cytotoxic stimulus [49]. Also, they potentiated the response of pancreatic carcinoma cells to several cytotoxic stimuli including gemcitabine, TRAIL or irradiation [49].

Exploiting the mitochondrial pathway for cancer therapy

The Bcl-2 family of proteins consists of both anti-apoptotic members, *e.g.* Bcl-2, Bcl-X_L, Mcl-1, as well as pro-apoptotic molecules

such as Bax, Bak, Bad and BH3 domain only proteins [13]. There are currently two alternative models how BH3-only proteins trigger activation of the multimeric molecules Bax and Bak. According to the direct activation model [63], direct activators, *i.e.* Bim and cleaved Bid (tBid), bind to Bax and Bak to trigger their activation, while BH3-only proteins that act as sensitizers, *e.g.* Bad, bind to the pro-survival Bcl-2 proteins. By comparison, the indirect activation model holds that BH3-only proteins activate Bax and Bak directly by engaging anti-apoptotic Bcl-2 proteins, thereby freeing up Bax and Bak [64, 65]. Bak has recently been reported to trigger apoptosis only if both Bcl-X_L and Mcl-1 are inactivated [66]. Imbalances in the ratio of anti-apoptotic *versus* pro-apoptotic Bcl-2 proteins with a relative increase in the anti-apoptotic molecules have been reported to several human cancers. In pancreatic carcinoma, it is especially Bcl-X_L that is detected at elevated levels.

To target aberrant expression of anti-apoptotic Bcl-2 related proteins, different strategies have been developed. One of the most promising approaches in this area is the development of a small molecule inhibitor that antagonizes Bcl-2, Bcl-X_L and Bcl-w [67]. To this end, a multidisciplinary effort to target the protein–protein interaction site between anti-apoptotic Bcl-2 proteins and Bax or Bak resulted in the generation of small molecule antagonists, which bind to the surface groove of Bcl-2, Bcl-X_L and Bcl-w that normally binds the BH3 domain of Bax or Bak [67]. ABT-737 is the prototypic compound that has been extensively studied in preclinical models [68]. By preventing the binding of these anti-apoptotic Bcl-2 proteins to Bax or Bak, the small molecule inhibitor can directly trigger apoptosis in some susceptible cell lines or sensitize cancer cells for apoptosis [67]. Recently, ABT-737 has been shown to synergistically enhance TRAIL-mediated cytotoxicity in pancreatic cancer cell lines by promoting the cross-talk between the extrinsic and intrinsic apoptotic pathways [69]. Further, the BH3 mimetic obatoclax that also antagonize Mcl-1 in addition to Bcl-2, Bcl-X_L and Bcl-w [70] has recently been reported to potentiate TRAIL-induced apoptosis in human pancreatic cancer cells [71]. An orally available second generation compound with improved pharmacokinetic properties, ABT-263, is currently under evaluation in early clinical trials in small-cell lung cancer and B-cell malignancies [72]. Also TW-37, another small-molecule inhibitor of Bcl-2, was shown to inhibit cell growth and invasion and increased apoptosis in pancreatic cancer [73]. Further, Bcl-X_L antisense oligonucleotides increased the sensitivity of pancreatic cancer cells to gemcitabine or irradiation [74–76].

Conclusions

Evasion of apoptosis is a hallmark of human cancers including pancreatic cancer that promotes tumorigenesis and also contributes to treatment resistance. Some of the molecular mechanisms causing resistance to apoptosis in pancreatic cancer have already been identified. Further, preclinical studies indicate that components of the apoptotic machinery may serve as targets for the development of experimental cancer therapies. The challenge now is to translate these concepts into a clinical application for the treatment of pancreatic carcinoma. Such apoptosis-based therapeutics may open new avenues to improve the poor outcome of patients with pancreatic cancer.

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