Cellular Signalling 28 (2016) 1652–1662

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Negative control of TRAIL-R1 signaling by transforming growth factor β1 in pancreatic tumor cells involves Smad-dependent down regulation of TRAIL-R1

David I. Radke [a], Hendrik Ungefroren [b], Ole Helm [a], Susann Voigt [c], Gökhan Alp [a], Hendrik Braun [a], Sebastian Hübner [a], Janine Dilchert [a], Susanne Sebens [a], Dieter Adam [c], Holger Kalthoff [a], Anna Trauzold [a,d,*]

[a] Institute for Experimental Cancer Research, University of Kiel, D-24105 Kiel, Germany
[b] First Department of Medicine, UKSH and University of Lübeck, D-23538 Lübeck, Germany
[c] Institute of Immunology, University of Kiel, D-24105 Kiel, Germany
[d] Clinic for General Surgery, Visceral, Thoracic, Transplantation and Pediatric Surgery, University Hospital Schleswig-Holstein, Campus Kiel, D-24105 Kiel, Germany

ARTICLE INFO

Article history:
Received 16 December 2015
Received in revised form 27 June 2016
Accepted 31 July 2016
Available online 01 August 2016

Keywords:
TRAIL-R1
TGFβ
Smad4
Apoptosis
Non-canonical TRAIL receptor signaling

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is characterized by both, overexpression of transforming growth factor (TGF)β and resistance of the tumor cells to many apoptosis-inducing stimuli. The latter negatively impacts the outcome of therapeutic efforts and represents one important mechanism which tumor cells utilize to escape the immune surveillance. Since TGFβ acts as a tumor promoter in advanced tumor stages and suppression of apoptosis is a known driver of tumor progression, it is possible that TGFβ functions as a crucial determinant of tumor cell sensitivity to apoptosis in PDAC. Here, we have studied the impact of TGFβ on TNF-related apoptosis inducing ligand (TRAIL)-induced signaling in PDAC cells.

In TGFβ-responsive Panc1 and Colo357 cells, TGFβ1 reduced total and plasma membrane-associated levels of TRAIL-R1 but not those of TRAIL-R2. Consistent with the known predominant role of TRAIL-R1 in TRAIL-mediated signaling in PDAC, TGFβ1 inhibited TRAIL-induced DISC formation and apoptosis as well as phosphorylation of MAPKs and IκBα. Similarly, it also reduced signaling of TRAIL-R1 following its specific activation with an agonistic antibody. In contrast, specific TRAIL-R2 signaling remained unchanged. The TGFβ1 effect on TRAIL-R1 expression was mimicked by ectopic expression of a kinase-active version of the TGFβ type I receptor ALK5 (ALK5-T204D) but not by ALK5 double mutant lacking the ability to phosphorylate Smad proteins (RImL45-T204D). Moreover, TGFβ regulation of TRAIL-R1 was absent in two PDAC cell lines lacking the Smad4 gene *DPC4* and siRNA-mediated silencing of Smad4 in Smad4-positive Panc1 cells abolished the TGFβ-mediated decrease in TRAIL-R1 expression, together showing that ALK5/Smad4 signaling is crucial for TGFβ regulation of TRAIL-R1 expression. Our results suggest a novel tumor-promoting function of TGFβ1. By downregulating TRAIL-R1, TGFβ1 may not only promote tumor escape from immune surveillance but also negatively impact on TRAIL- or TRAIL-R1-based therapy regimens for treatment of PDAC.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a cancer entity with an extremely poor prognosis and surgery (R0 resection) being the only curative therapeutic option so far. However, most patients harbor metastatic lesions already at the time of diagnosis, a condition which precludes surgical intervention [1,2]. Resistance to chemotherapy as a consequence of a generalized apoptosis resistance of tumor cells is a predominant feature of PDAC and poses a great clinical problem in the treatment of this tumor entity.

Amongst other growth factors such as EGF and PDGF, PDACs frequently express all three isoforms (1, 2, 3) of transforming growth factor (TGF)β [3]. TGFβ1 plays a crucial role in tumor cell behavior by controlling cell growth, angiogenesis, epithelial-mesenchymal

Abbreviations: ALK5, activin receptor-like kinase 5; Bcl-xL, B-cell lymphoma-extra large; DISC, death inducing signaling complex; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FADD, Fas-associated protein with death domain; FLIP, FLICE-like inhibitory protein; JNK, c-JUN N-terminal kinase; Lexa, Lexatumumab; Mapa, Mapatumumab; Mcl-1, myeloid cell leukemia 1; MFI, median fluorescene intensity; NF-κB, nuclear factor κB; PDAC, pancreatic ductal adenocarcinoma; PDGF, platelet derived growth factor; PKC, protein kinase C; TGFβ, transforming growth factor β; TRAIL, TNF-related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

* Corresponding author at: Institute for Experimental Cancer Research, University of Kiel, Clinic for General Surgery, Visceral, Thoracic, Transplantation and Pediatric Surgery, University Hospital Schleswig-Holstein, Campus Kiel, D-24105 Kiel, Germany.
E-mail address: atrauzold@email.uni-kiel.de (A. Trauzold).

http://dx.doi.org/10.1016/j.cellsig.2016.07.016
0898-6568/

transition (EMT), tumor cell migration, invasion, and metastasis (reviewed by [4]). Current data indicate a dual function of TGFβ1 in tumorigenesis. In normal cells and in cancer cells in early stages of tumor development, TGFβ1 acts as a tumor suppressor. In contrast, in late stages of tumor progression, it functions as a tumor promoter, enhancing immune suppression, angiogenesis, migration, invasion and metastatic dissemination [4]. The three TGFβ isoforms signal via a heterotetrameric complex consisting of two transmembrane receptor serine/threonine kinases, one of type I (TβRI, also known as activin receptor-like kinase 5, ALK5) and one of type II (TβRII). Signal transduction is initiated by binding of TGFβ to TβRII resulting in the recruitment of ALK5 into the complex and its activation through phosphorylation in the GS region (glycine/serine rich domain). This triggers the phosphorylation of intracellular mediators, the receptor-regulated Smads (R-Smads) Smad2 and Smad3 through the L45 loop in ALK5. Phosphorylated Smad2 or Smad3 then complex with tumor suppressor Smad4/DPC4 and the resulting hetero-Smad complex translocates to the nucleus to activate the transcription of various TGFβ-responsive genes [4].

The role of TGFβ-signaling as a tumor suppressor pathway in PDAC is best illustrated by the fact that 55% of pancreatic tumors harbor chromosomal deletions or mutations in Smad4 (also termed *DPC4)* [5], a tumor suppressor mediating TGFβ-dependent growth inhibition [6] and anti-angiogenesis [7]. The loss-of-function mutations in Smad4 or other disturbances in Smad-signaling [8] eventually result in loss of tumor suppression and gain of resistance of tumors towards the antiproliferative and pro-apoptotic effects of TGFβ. Consequently, pancreatic cancer cell-derived TGFβs cannot suppress the growth/survival of the cancer cells, but instead may promote invasion and metastasis through alternate pathways [9,10]. The crucial role of the Smad pathway in PDAC-malignancy was also highlighted by results from orthotopic xenotransplantation experiments with TGFβ responsive Panc1 cells, demonstrating that Smad-signaling activated by a kinase-active mutant of ALK5 suppressed primary tumor growth, but enhanced metastatic progression [11].

The death receptors TRAIL-R1 and TRAIL-R2 are able to induce cell death when cross-linked by their cognate ligand TRAIL. TRAIL has attracted particular attention due to its apparent selectivity for inducing apoptosis in tumors, while sparing normal healthy cells [12,13]. Therefore, TRAIL and agonistic TRAIL-R1 and TRAIL-R2 specific antibodies are currently evaluated in clinical trials for the treatment of different malignancies [14]. However, there are several important concerns limiting TRAIL-usage as a therapy option for PDAC. First, PDAC cells are mostly apoptosis resistant [15,16]. Second, beside their pro-apoptotic function, both TRAIL-R1 and TRAIL-R2 are able to induce several non-apoptotic signaling pathways leading to tumor cell migration, invasion and metastasis in vivo [17–19]. Third, plasma membrane expression of TRAIL-R1 and TRAIL-R2 is strongly diminished in PDAC tissue compared to the adjacent normal pancreatic tissue [20]. In contrast, the intracellular expression of TRAIL-R2 is enhanced and for nuclear TRAIL-R2, a protumoral miRNA-associated function has been described recently [21]. Characteristic for PDAC is the well-developed tumor stroma containing large amounts of TGFβ [22]. Although many studies separately investigated TGFβ or TRAIL death receptor functions in PDAC, we are not aware of any study that addressed the potential interdependence of these highly tumor cell-relevant pathways in PDAC. In the present work, we therefore analyzed the effects of TGFβ on TRAIL receptor expression and signaling in pancreatic tumor cells.

We show here that treatment of PDAC cells with TGFβ1 specifically attenuated TRAIL-R1-dependent responses such as induction of apoptosis and pro-inflammatory signaling controlling cytokine expression, invasion and metastasis. Mechanistically, TGFβ1 decreased the levels of TRAIL-R1, an effect that required activation of the canonical Smad pathway. These findings may have far-reaching implications as TRAIL-mediated functions may be blunted in a TGFβ1-rich tumor microenvironment which is characteristic for PDAC.

2. Material and methods

2.1. Cell culture and stimulation

The pancreatic cancer cell lines Panc1, Colo357, Capan1 and Hs766T were cultured as previously described [23]. Stably retrovirally transduced Panc1 cells (TJBA5bMoLink-neo empty vector, ALK5-T204D, RImL45-T204D) were cultured in RPMI 1640 media supplemented with 10% FCS, 1 mM GlutaMAX and 1 mM sodium pyruvate (LifeTechnologies, Darmstadt, Germany). For stimulation, cells were seeded in culture plates (1.5×10^5/well in 6-well plates or 5×10^3/well in 96-well plates), allowed to adhere for 24 h and exposed in medium with reduced FCS content (1%) to TGFβ1 (ReliaTech, Wolfenbüttel, Germany, 10 ng/ml) for 72 h. To study TRAIL receptor signaling cells were treated with Mapatumumab (10 μg/ml), Lexatumumab (10 μg/ml), both kindly provided by Robin Humphreys Human Genome Sciences, Rockville, MD, USA, or with recombinant human TRAIL (50 ng/ml, PeproTech, Hamburg, Germany) for additional 3 h or 24 h. To neutralize the biological activity of the endogenous, tumor cell-derived TRAIL, cells were cultured in the presence of a neutralizing anti-TRAIL antibody (1 μg/ml, MAB375, R&D Systems, Wiesbaden-Nordenstadt, Germany). To knock down the expression of Smad4, Panc1 cells were transiently transfected with the Smad4-specific siRNA (12 nM, GeneSolution siRNA, Qiagen, Hilden, Germany; Hs_SMAD4_4, Hs_SMAD4_5, Hs_SMAD4_6) or with the control siRNA (AllStars Negative Control, 12 nM, Qiagen) using HiperFect reagent (Qiagen).

2.2. DISC analysis

Immunoprecipitation of the DISC of TRAIL death receptors was performed with TRAIL-Flag-Fc kindly provided by H. Walczak (Imperial College, London, UK). One 80%-confluent 175-cm^2 flask of cells was used per condition. Cells treated with TGFβ1 (72 h, 10 ng/ml) or untreated cells were stimulated with 1 μg/ml Flag-TRAIL for 1 h–3 h at 37 °C or left untreated, washed twice with ice-cold PBS and lysed with 1.5 ml lysis buffer (30 mM Tris–HCL, pH 7.5, 1% Triton X-100, 10% glycerol, 120 mM NaCl) supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) for 20 min on ice. After centrifugation (30 min, 14,000 ×g) the DISC was precipitated with M2-conjugated agarose beads (Sigma Aldrich, Taufkirchen, Germany) overnight at 4 °C. TRAIL-Flag-Fc (0.1 μg) was added to the lysates from unstimulated cells together with M2-conjugated agarose beads. The precipitates were washed five times with ice-cold lysis buffer. Bound proteins were eluted by incubation at 70 °C for 10 min in Laemmli buffer.

2.3. Flow cytometric detection of TRAIL receptors

The cell surface expression of TRAIL receptors was determined as described previously [23]. Antibodies against TRAIL-R1 and TRAIL-R2 (anti-TRAIL-R1 (mouse mAb clone HS101) and anti-TRAIL-R2 (mouse mAb clone HS201)) were purchased from Alexis, (Heidelberg, Germany). Controls were incubated with appropriate isotype matched antibodies (IgG1 mouse mAb clone 11711; R&D Systems) and labeled with the corresponding secondary antibodies (biotin conjugated anti-mouse IgG (goat Ab, Sigma Aldrich St. Louis, MO, USA) and Streptavidin PE (Becton Dickinson, Heidelberg, Germany)). Measurements were performed with a FACSCalibur (Beckton Dickinson), equipped with dual lasers (488 nm argon and 633 nm diode). Data analysis was performed using Weasel 3.1 software (The Walter and Eliza Hall Institute of Medical Research, Victoria, AUS).

2.4. Cell death assays

Cells were seeded in culture plates (1.5×10^5/well in 6-well plates (5×10^3/well in 96-well plates), allowed to adhere for 24 h, exposed