Orai1 and STIM1 mediate SOCE and contribute to apoptotic resistance of pancreatic adenocarcinoma

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ABSTRACT

The store-operated calcium channels (SOCs) represent one of the major calcium-entry pathways in non-excitable cells. SOCs and in particular their major components Orai1 and STIM1 have been shown to be implicated in a number of physiological and pathological processes such as apoptosis, proliferation and invasion. Here we demonstrate that Orai1 and STIM1 mediate store-operated calcium entry (SOCE) in pancreatic adenocarcinoma cell lines. We show that both Orai1 and STIM1 play pro-survival anti-apoptotic role in pancreatic adenocarcinoma cell lines, as siRNA-mediated knockdown of Orai1 and/or STIM1 increases apoptosis induced by chemotherapy drugs 5-fluorouracil (5-FU) or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line via upregulation of Orai1 and STIM1. Altogether our results reveal the novel calcium-dependent mechanism of action of the chemotherapy drugs 5-FU and gemcitabine and emphasize the anti-apoptotic role of Orai1 and STIM1 in pancreatic adenocarcinoma cells. This article is part of a Special Issue entitled: Calcium signaling in health and disease. Guest Editors: Geert Bultynck, Jacques Haiech, Claus W. Heizmann, Joachim Krebs, and Marc Moreau.

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1. Introduction

Pancreatic cancer and in particular pancreatic ductal adenocarcinoma (PDAC) representing the most prevalent pancreatic neoplasm accounting for about 90% of all pancreatic tumors [1,2], is one of the leading causes of cancer death in men and women [3]. The current five-year relative survival rate is about 6% whereas most patients die within the one year following cancer detection [2]. One of the reasons of this is that early stage pancreatic cancer usually has no symptoms and thus the majority of cases are diagnosed at the late metastatic or invasive stages which are not suitable for surgery. The lack of useful early stage diagnostic markers and techniques further worsen the situation [2]. Furthermore, high resistance of PDAC to conventional radiotherapy and chemotherapy treatments contributes to the pessimistic prognosis of this disease [4–6].

Pancreatic cancer cells have been shown to exhibit a number of genetic mutations leading to uncontrolled cell proliferation, acquisition of metastatic potential as well as evasion of programmed cell death (apoptosis) [7]. Apoptosis is a physiological process that is essential for normal tissue homeostasis and deregulation of apoptotic machinery has been shown to contribute to the malignant transformation. Evading apoptosis represents one of the cancer hallmarks initially defined by Hanahan and Weinberg [8]. Indeed, resistance to apoptosis has been implicated in moderate efficiency or failure of a number of anti-cancer treatments. Thus, targeting apoptosis represents a promising strategy in cancer treatments.

Numerous studies have emphasized the key role of calcium and calcium-permeable ion channels in the regulation of apoptosis [9–12]. The store-operated calcium channels (SOCs) represent one of the major calcium-entry pathways in non-excitable cells. SOCs and in particular their major components Orai1 and STIM1 [13] have been shown to be implicated in a number of physiological processes, including proliferation, development, contraction and motility [14–19]. Moreover, recent studies suggest an important role for Orai1 and STIM1 in cancerogenesis. Both Orai1 and STIM1 have been reported to be involved in the regulation of apoptosis, proliferation, migration and invasion in a number of human cancers, including prostate cancer, breast cancer, cervical cancer and glioblastoma [12,20–28].

Here we demonstrate that Orai1 and STIM1 mediate store-operated calcium entry (SOCE) in pancreatic adenocarcinoma cell lines. We show that both Orai1 and STIM1 play pro-survival anti-apoptotic role in
pancreatic adenocarcinoma cell lines, as siRNA-mediated knockdown of ORAI1 and/or STIM1 increases apoptosis induced by chemotherapy drugs 5-fluorouracil (5-FU) or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line via upregulation of ORAI1 and STIM1.

Altogether our results reveal the novel calcium-dependent mechanism of action of the chemotherapy drugs 5-FU and gemcitabine and emphasize the anti-apoptotic role of ORAI1 and STIM1 in pancreatic adenocarcinoma cells.

2. Materials and methods

2.1. Antibodies and reagents

Mouse anti-ORAI1 (PM-5205, ProSci), mouse anti-STIM1 (610954, BD Transduction), mouse anti-Actin (A5441, Sigma). 5-Fluorouracil (5-FU) (F6627) was from Sigma. Gemcitabine (sc-204763A) was from Santa Cruz Biotechnology. Thapsigargin (TG) (TG1138) was from Tocris.

2.2. Cell culture and transfection

Pancreatic adenocarcinoma cell line Panc1 from the American Type Culture Collection (ATCC) was cultured in Dulbecco's minimal essential medium DMEM + GlutaMAX (31966, Invitrogen, Life Technologies Inc.) supplemented with 10% FCS (PAA Gold). Pancreatic adenocarcinoma cell lines ASPC1 and BxPC3 from the ATCC were cultured in RPMI 1640 medium (31870, Gibco-Life Technologies) supplemented with 2.5% Horse Serum (S 9135, Biochrom) and 10% FCS (PAA Gold). Pancreatic adenocarcinoma cell line MiaPaca2 from the ATCC was cultured in DMEM/F12 medium (31330, Gibco-Life Technologies) supplemented with 2.5% Horse Serum (S 9135, Biochrom) and 10% FCS (PAA Gold). Pancreatic adenocarcinoma cell line Capan1 from the ATCC was cultured in IMDM medium (SH 30229.01 HyClone, Thermoscientific) supplemented with 20% FCS (PAA Gold). Immortalized human pancreatic ductal epithelial cells H6C7 were obtained from Dr. Ming-Sound Tsao and cultured in KBM medium (CC-3101, Lonza) supplemented with KGM SingleQuots (CC-4131, Lonza). Cells were grown at 37 °C in an humidified atmosphere containing 5% CO2.

Cells were transfected with 40 nM of siRNA against ORAI1, STIM1 or both (Dharmacon Inc., Fremont, CA, USA) using Hyperfect transfection reagent (Qiagen Inc.), following the manufacturer's instructions. siRNA sequences were the following: CT: 5′-CUCUAACCCUGAUAUCUCGA (dTdT)-3′, hOrai1: 5′-UGACCAAGUGCCCAACUUC (dTdT)-3′, hSTIM1: 5′-GCCUCUGAUAACUGCUC (dTdT)-3′.

2.3. qRT-PCR

Total RNA was extracted using TRI reagent (Sigma) and treated with RNase-free DNase (Ambion). cDNA was synthesized by reverse transcription. qRT-PCR was performed in a real-time thermal cycler Cfx C1000 (Bio-Rad). Primers are listed in Table 1. PCR was performed in a real-time thermal cycler Cfx C1000 (Bio-Rad). The lysates were centrifuged at 15,000 × g at 4 °C for 15 min to remove cell debris and supernatant protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology). 30 μg of total protein was subjected to SDS-PAGE followed by transfer to PVDF membranes using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The membranes were blocked in a 5% fat-free milk containing TNN buffer (Tris–HCl, pH 7.5, 140 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were next incubated overnight at 4 °C with primary antibodies, and then for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase. After washing, the membranes were processed for chemiluminescence detection using Luminata Western HRP substrate (Milipore). Image J software was employed for quantitative analysis.

2.5. Calcium imaging

Pancreatic cancer cells were grown on glass coverslips to carry out calcium imaging experiments. Ratiometric dye Fura-2/AM (F1221, Invitrogen) was used as a Ca2+ indicator. Cells were loaded with 2 μM Fura-2/AM for 45 min at 37 °C and subsequently washed three times with external solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 5 glucose, 10 hepes (pH 7.4). The coverslip was then transferred in a perfusion chamber on the stage of Nikon Eclipse Ti microscope. Fluorescence was alternatively excited at 340 and 380 nm with a monochromator (Polychrome IV, TILL Photonics GmbH) and captured at 510 nm by a QImaging CCD camera (QImaging). Acquisition and analysis were performed with the MetaFluor 7.7.5.0 software (Molecular Devices Corp.).

2.6. Apoptosis assays

The level of apoptosis was determined by Hoechst staining. Cells were grown on 6-well plates and transfected with siCT, siORAI1 or siSTIM1. In 24 h cell were subjected to chemotherapy treatments for another 72 h. At the end of the treatments, both floating and attached cells were collected by trypsinization, centrifuged and resuspended in 1 ml of phosphate-buffered saline (PBS). Following cytospin cells were fixed with ice-cold methanol for 10 min, washed with PBS and stained with 5 μg/ml Hoescht 33258 for 10 min at room temperature. Cells were then washed twice with PBS, mounted in glycerogel (DAKO) and subjected to fluorescence microscopy analysis. Nuclear morphology was displayed on a Zeiss Axioscope A1 fluorescence microscope (405–435 nm). The percentage of apoptotic cells (with condensed/fragmented nuclei) was determined by counting at least 500 cells in random fields.

Alternatively, apoptotic cells were detected by Alexa Fluor® 488 Annexin V/Propidium iodide double staining. At the end of the treatments, both floating and attached cells were collected by trypsinization, centrifuged, washed with PBS and stained with Alexa Fluor® 488 Annexin V and Propidium iodide according to the manufacturer's instructions (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Life Technologies). Cells were examined by fluorescence microscopy on a Zeiss Axioscope A1 microscope. The percentage of Alexa Fluor® 488 Annexin V-positive cells was determined by counting at least 500 cells in random fields.

2.7. Data analysis

Data were analyzed using Origin 7.0 (Microcal Software). Statistical analysis was performed using Student's t-test, and p < 0.05 was considered as significant. Asterisks denote: *p < 0.05, **p < 0.01 and ***p < 0.001.
3. Results

3.1. ORAI1 and STIM1 are differentially expressed in pancreatic cancer cells

Although both ORAI1 and STIM1 have been previously shown to be expressed in a variety of cell types, the information about their expression in PDAC cell lines is missing. Therefore, we first sought to examine if they are present in several available PDAC cell lines, namely Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 as well as in “normal” immortalized human pancreatic ductal epithelial cells H6C7. By using qRT-PCR technique we found that ORAI1 and STIM1 are differentially expressed in all the cell lines tested (Fig. 1A). Further, we characterized the expression of ORAI1 and STIM1 on the protein level by Western blotting. We found that both ORAI1 and STIM1 are expressed on the protein level in all the cell lines and the levels of their expression differ between the cell lines, which correlates well with qRT-PCR data (Fig. 1B and C). For the subsequent experiments, we decided to focus on Panc1 cell line, as it is characterized by relatively high levels of ORAI1 and STIM1 expression.

3.2. ORAI1 and STIM1 are involved in SOCE in pancreatic cancer cells

Numerous reports demonstrated that ORAI1 and STIM1 mediate calcium release-activated channel (CRAC) activity and thus SOCE in a variety of cell types. Given that this function of ORAI1 and STIM1 has never been demonstrated in PDAC cells, we next investigated if ORAI1 and STIM1 are involved in SOCE in these cells by using siRNA approach. Panc1 cells were transfected with siCT, siORAI1 or siSTIM1. We determined the efficiency of siRNA transfections in 48 h on mRNA and protein levels by using qRT-PCR and Western blotting techniques respectively (Fig. 2C and D). 48 h following siRNA transfection we tried to record IP3-induced CRAC activity by performing electrophysiological experiments in Panc1 cells. In most cases IP3 dialysis did not induce any recordable CRAC current, thus precluding any statistical analysis of the effect of ORAI1/STIM1 knockdown. Therefore, we moved to an approach based on calcium imaging using Fura2/AM probe. 48 h following siRNA transfection cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. We checked whether siRNA-mediated knockdown of ORAI1 or STIM1 influences

![Fig. 1. ORAI1 and STIM1 are differentially expressed in pancreatic cancer cells. (A) qRT-PCR detection of expression of ORAI1 and STIM1 in H6C7, Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 cell lines. Expression levels of ORAI1 and STIM1 relative to GAPDH for each cell line were normalized to ORAI1 level in “normal” H6C7 cell line. Data presented as means ± S.D. n = 3. (B) Western blot showing the expression of ORAI1 in H6C7, Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 cell lines. Densitometric quantitations for normalized ORAI1 relative to Actin are shown. Bar plot shows the quantification of the protein expression levels of ORAI1 relative to Actin for each cell line normalized to ORAI1 level in H6C7 cell line. Data presented as means ± S.D. n = 3. (C) Western blot showing the expression of STIM1 in H6C7, Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 cell lines. Densitometric quantitations for normalized STIM1 relative to Actin are shown. Bar plot shows the quantification of the protein expression levels of STIM1 relative to Actin for each cell line normalized to STIM1 level in H6C7 cell line. Data is represented as means ± S.D. n = 3.](image-url)
cytosolic calcium levels in Panc1 cells using thapsigargin (TG), an inhibitor of sarco/endoplasmic reticulum Ca2+-ATPase (SERCA). More specifically, intracellular stores were depleted by TG in nominally calcium free extracellular saline followed by addition of 2 mM Ca2+ to the cells to initiate the influx via store-operated calcium channels. When this assay was performed on siORAI1- or siSTIM1-transfected Panc1 cells, calcium entry was significantly reduced (Fig. 2A and B). Further, ORAI1 and STIM1 knockdown also caused a decrease in SOCE in other cell lines tested, namely ASPC1, MiaPaca2 and Capan1 (Fig. S1A, S1B and S1C). These results suggested that ORAI1 and STIM1 mediate SOCE in PDAC cell lines. In addition, TG-stimulated calcium entry was completely inhibited by La3+(10 μM), Gd3+(10 μM) or BTP2 (10 μM), exhibiting pharmacological properties characteristic of SOCE (Fig. S2A and S2B).

3.3. Downregulation of ORAI1 and STIM1 sensitizes Panc1 cells to chemotherapy treatments

Previous reports indicated that ORAI1, STIM1 as well as SOCE play an important role in cell physiology and pathology. In particular, their role in apoptosis regulation has been extensively studied in different cell types. Therefore, we next investigated whether ORAI1 and STIM1 are involved in apoptosis regulation in Panc1 cells. Cells were transfected with siCT, siORAI1, siSTIM1 or siORAI1 + siSTIM1. In 24 h cells were treated with chemotherapy drugs 5-FU or gemcitabine (GB) for 72 h. At the end of treatments cells were collected, stained with Hoechst and Annexin V/PI and subjected to fluorescence microscopy analysis of nuclear morphology. Alternatively, apoptotic cells were detected by Annexin V/PI staining.
Downregulation of ORAI1 or/and STIM1 did not induce significant changes to the basal level of apoptosis in untreated cells (Fig. 3A and B). However, when cells were treated with 5-FU or gemcitabine, the downregulation of ORAI1 or/and STIM1 increased the levels of apoptosis induced by these drugs (Fig. 3A and B). These results suggested that ORAI1 and STIM1 play pro-survival anti-apoptotic role in Panc1 cells.

3.4. Chemotherapy drugs 5-FU and gemcitabine increase SOCE in Panc1 cells

The regulation of chemotherapy-induced apoptosis by ORAI1 and STIM1 suggests that apparently there is a link between chemotherapy drugs and Ca^{2+}-homeostasis. To test this assumption we incubated Panc1 cells in basal medium with or without chemotherapy agents (5-FU (50 μM) or gemcitabine (50 μM)) for 24 h. At the end of the treatment cells were loaded with Fura2/AM probe and subjected to calcium imaging experiment. We tested whether chemotherapy treatments influence cytosolic calcium levels in Panc1 cells using the same TG-based approach as described above. Interestingly, we have found that both 5-FU and gemcitabine treatments significantly increased SOCE in these cells (Fig. 4A and B). We hypothesized that this effect can be the consequence of the increase in expression levels of SOCs by chemotherapy agents. To test this hypothesis, we next assessed the expression levels of ORAI1 and STIM1 following 24 h treatment with 5-FU or gemcitabine. 5-FU and gemcitabine significantly increased the expression of ORAI1 and STIM1 on both mRNA and protein levels (Fig. 4C and D). These results correlate well with calcium imaging data (Fig. 4A and B), suggesting that chemotherapy agents 5-FU and gemcitabine can influence cytosolic calcium levels by upregulation of ORAI1 and STIM1.

4. Discussion

In this study, we provide evidence that ORAI1 and STIM1 mediate SOCE in pancreatic adenocarcinoma cell lines. We show that both ORAI1 and STIM1 play pro-survival anti-apoptotic role in pancreatic adenocarcinoma cell lines, as siRNA-mediated knockdown of ORAI1 and/or STIM1 increases apoptosis induced by chemotherapy drugs 5-FU or gemcitabine. We also demonstrate that both 5-FU and gemcitabine treatments increase SOCE in Panc1 pancreatic adenocarcinoma cell line apparently via upregulation of ORAI1 and STIM1.

Numerous studies demonstrate the important role of SOCE in a plethora of cellular processes and functions in different cell types, including endothelial cell proliferation [15], smooth muscle migration [16,17] and skeletal muscle development and contraction [18]. Moreover, SOCE has been implicated in a number of pathological processes typical for cancer, such as breast tumor cell migration and metastasis [29], human glioblastoma invasion [26] and apoptosis in a variety of cell types [22–24,30]. Therefore, the identification of the molecular nature of SOCs in PDAC is of great importance as it can reveal novel approaches for treating pancreatic cancer through targeting SOCE-dependent processes.

The evidence presented here clearly demonstrates that ORAI1 and STIM1 are expressed in five PDAC cell line tested namely Capan1, ASPC1, Panc1, MiaPaca2 and BxPC3 as well as in “normal” human pancreatic ductal epithelial cells H6C7. It should be noted, that “normal”
H6C7 cells show relatively low levels of ORAI1 and STIM1 expression compared to several cancer cell lines, in particular Capan1 and Panc1. This result indirectly suggests that these cancer cells upregulate ORAI1 and STIM1 to protect themselves against apoptosis.

Interestingly, despite the suggested important role of SOCs in the regulation of apoptosis, metastasis and invasion in different cancers [22–24,26,29,30], their role in PDAC cells have never been studied.

Our results revealed the anti-apoptotic role of ORAI1 and STIM1 in Panc1 cells. We showed that siRNA-mediated knockdown of ORAI1 and/or STIM1 increases apoptosis induced by 5-FU or gemcitabine in Panc1 cells.

Previous studies reported conflicting results in regard to the role of SOCE, ORAI1 and STIM1 in apoptosis regulation. Some reports suggest that SOCE, ORAI1 as well as STIM1 contribute to apoptosis induced by various stress stimuli [22,30], while others demonstrate their pro-survival anti-apoptotic role [23,24,31]. Indeed, ORAI1 was reported to contribute to the establishment of an apoptosis-resistant phenotype in prostate cancer cells and ORAI1 knockdown protected LNCaP cells against TG- or oxaliplatin/cisplatin-induced apoptosis [22]. In line with this, pharmacological SOCE inhibition or STIM1 knockdown was demonstrated to inhibit hydrogen peroxide-induced apoptosis in HT22 cells via alleviation of intracellular Ca2+ overload, restoration of the mitochondrial membrane potential and decrease of cytochrome C release [32]. In contrast, pharmacological inhibition of SOCE or STIM1 downregulation was shown to enhance apoptosis induced by cisplatin in non-small cell lung cancer cells [31]. Further, it was reported that ORAI1-driven Ca2+ –entry delays the induction of the CD95-mediated apoptotic signal in leukemic T-cell lines through the translocation of the Ca2+ –dependent protein kinase C (PKC) z to the death-inducing signaling complex and its subsequent inactivation in T-cells. This prevented CD95-mediated caspase activation and delayed delivery of the apoptotic signal [23].

Although it seems that these studies represent contradictory results, there are several clues which can explain this discrepancy. First, it is well known that different cells are characterized by a different set and level of expressed genes, in particular ORAI1 and STIM1 [14]. Thus, the contradiction could arise from the different cell types used in these studies. Second, both ORAI1 and STIM1 were shown to regulate the activity of a number of intracellular effectors including PKC z [23], PKC δ [33], extracellular signal–related kinases 1 and 2 (ERK 1/2) [34], calpains as well as cytoplasmic kinase Pyk2 [21]. Further, the expression/function of ORAI1 and STIM1 was reported to be regulated by several protein kinases, such as serum and glucocorticoid-inducible kinase 1 (SGK1) and AMP activated kinase (AMPK) [35], by transcription factor nuclear factor κB (NF-κB) [36] as well as by cytoskeleton reorganization [37]. Moreover, both ORAI1 and STIM1 were shown to have SOCE independent functions. It was reported that ORAI1 could stimulate mammary tumorigenesis by store- and STIM1-independent pathway involving secretory pathway Ca2+ –ATPase, SPCA2 [38]. Likewise, STIM1 was suggested to be a general stress sensor [39] capable of interacting with multiple molecular targets, including ORAI1 [13], TRPC1 [40] and Cav1.2 channels [41]. Considering such a complex regulatory network and the proven role for many of ORAI1/STIM1 targets in both pro-survival and pro-apoptotic processes, the role of SOCE, ORAI1 and STIM1 in each particular cell type could differ depending on the signaling pathway activated.

We noticed that STIM1-knockdown increased chemotherapy-induced apoptosis more effectively than ORAI1-knockdown, although the difference did not reach statistical significance. This result points to the potential ORAI1-independent role of STIM1 in apoptosis regulation in Panc1 cells.

Further, the effect of ORAI1 - and/or STIM1-knockdown on apoptosis in Panc1 cells was more pronounced in the case of treatment with gemcitabine compared to 5-FU. This result could point to increased calcium-dependency of gemcitabine mechanisms of action compared to 5-FU. Interestingly, our results also suggest that both 5-FU and gemcitabine treatment increases SOCE in Panc1 cells and upregulate ORAI1 and STIM1. This result suggests that chemotherapeutic treatments could have calcium-dependent effects, which are unrelated to the primary DNA-targeting mechanisms of their action. Moreover, these calcium dependent effects could potentially contribute to the final efficacy of the drugs in cancer therapy. Further experiments are needed to understand the mechanism of this effect. We hypothesize that during chemotherapy treatments cells upregulate SOCs to resist apoptosis. Thus, the downregulation of SOCs in these conditions makes cells more sensitive to apoptosis induction.

In conclusion, given the important role of ORAI1 and STIM1 in a number of cellular processes and functions in different cell types, they could be potentially considered as a promising target in anti-cancer therapy. However, the vast heterogeneity in the reported roles for ORAI1 and STIM1 in different cell types confirms that their final effect on apoptosis could depend on multiple factors, such as cell type, nutrient and growth factor availability, pathology (cancer, inflammation, etc.) as well as intracellular signaling pathways involved. Therefore, future research is indispensable to better understand the specific mechanisms of apoptosis regulation by SOCE, ORAI1 and STIM1 to finally conclude if their modulators could be effective in cancer treatment in each particular case.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbmbamcr.2014.02.012.

References


