Knockdown of glucose-regulated protein 78 enhances poly(ADP-ribose) polymerase cleavage in human pancreatic cancer cells exposed to endoplasmic reticulum stress

XIA JIANG1, TATSUO KANDA1, SHINGO NAKAMOTO1,2, YUKI HAGA1, REINA SASAKI1, MASATO NAKAMURA1, SHUANG WU1, RINTARO MIKATA1 and OSAMU YOKOSUKA1

Departments of 1Gastroenterology and Nephrology, and 2Molecular Virology, Chiba University, Graduate School of Medicine, Chuo-ku, Chiba 260-8677, Japan

Received August 7, 2014; Accepted September 25, 2014

DOI: 10.3892/or.2014.3533

Abstract. The present study examined the expression of glucose-regulated protein 78 (GRP78/Bip) in human pancreatic cancer cell lines and the effect of knockdown of GRP78 on the cleavage of poly(ADP-ribose) polymerase (PARP). Human pancreatic cancer cell lines (KP-2, MIAPaCa-2, Panc-1 and SUIT-2), constitutively expressed GRP78. We also demonstrated that ER stress induced by thapsigargin upregulated protein levels of GRP78. In the presence of thapsigargin, knockdown of GRP78 enhanced the PARP cleavage in the human pancreatic cancer cells. These results provide evidence that GRP78 is a potential therapeutic target for ‘difficult-to-treat’ pancreatic cancer, in which ER stress signaling in part falls into disorder.

Introduction

Pancreatic cancer is almost the deadliest of all malignancies (1). In Japan, pancreatic cancer is currently the fifth leading cause of cancer-related death among individuals of both genders (2,3). Resection surgery is still the only potentially curative treatment for pancreatic cancer, and recent improvements in operative technique have been reported (4). Although advances in adjuvant treatment have been observed (5), in general, the prognosis of patients with pancreatic cancer is still poor. Further studies of the mechanisms of pancreatic carcinogenesis and cancer development are needed, and new therapeutic options are highly desirable.

Endoplasmic reticulum (ER) stress response in tumor cells is critical for tumor cell growth and cancer progression (6). The ER stress response is mediated by at least three sensor molecules: inositol-requiring enzyme 1α (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which are usually associated with glucose-regulated protein 78 (GRP78/Bip) (7). ER stress, which is associated with the accumulation of unfolded proteins, induces unfolded protein response (UPR), yet if ER stress is overloaded, cells could face death such as by apoptosis and autophagy. Downstream of IRE1α and PERK, the effector molecules, X-box-binding protein 1 (XBP1) and C/EBP homologous protein (CHOP), and growth arrest and DNA damage gene 34 (GADD34) all exist, and they are activated by ER stress. ER stress also leads to the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (8). For example, p90ATF6 is converted to the activated form p50ATF6, and p50ATF6 translocates to the nucleus (9). Basic leucine-zipper family factors p50ATF6 and XBP1 could induce expression of a subset of UPR-related genes, which include ER stress elements, and are involved in efficient protein folding, maturation and degradation in the ER (6).

The association between ER stress response and tumor growth and progression has been reported (10). We and others have reported that GRP78 is involved in cancer development and innate immune response in the liver (11-14). Liver and pancreas progenitors commonly develop from endoderm cells in the embryonic foregut (15). Pancreatic epithelial cells have a highly developed ER due to a strong engagement in digestive enzyme secretion (16). GRP78 is the main target of UPR signaling that promotes pancreatic cancer cell survival (17). GRP78 is involved in cancer progression as well as drug resistance (18,19). Hence, to decrease the ability of pancreatic cancer cells to survive and proliferate, it may be necessary to block GRP78 expression (17).

We previously demonstrated that blocking of the induction of UPR, as well as inhibition of GRP78 expression is associated with the cleavage of poly(ADP-ribose) polymerase (PARP) (13). In the present study, we examined the expression of ER stress-related molecules in human pancreatic cancer cell lines in the presence or absence of thapsigargin, one of the ER stress-inducers. We also investigated whether knockdown of GRP78 by small interfering RNA (siRNA) enhances the PARP cleavage in human pancreatic cancer cell lines exposed to ER stress.

Correspondence to: Dr Tatsuo Kanda, Department of Gastroenterology and Nephrology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan
E-mail: kandat-cib@umin.ac.jp

Key words: apoptosis, ER stress, GRP78/Bip, pancreatic cancer, resistance, UPR
Materials and methods

Cell culture. Human pancreatic cancer cell lines (KP-2, MIAPaCa-2, Panc-1 and SUIT-2) were grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Inhibitor of sarcoplasmic/endoplasmic reticulum (ER) Ca²⁺ ATPases (SERCA), thapsigargin, control siRNA (si-control) and siRNA for GRP78 (si-GRP78) were purchased from BioVision (Milpitas, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Western blotting. Twenty-four hours after thapsigargin (1 µM) treatment, cells were lysed in sodium dodecyl sulfate sample buffer, and after sonication, lysates were processed for western blot analysis (11). Briefly, protein samples were subjected to electrophoresis on 5-20% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). Membranes were probed with antibodies specific for ATF4, ATF6 and tubulin (Abcam, Cambridge, UK); GADD34, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and XBP1 (Santa Cruz); eIF2α, phospho-eIF2α (Ser51), GRP78/Bip and PARP (Cell Signaling Technology, Tokyo, Japan). After washing with PBS-T, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Signals were detected by means of enhanced chemiluminescence (GE Healthcare, Tokyo, Japan) and scanned by image analyzer LAS-4000 and Image Gauge (version 3.1) (Fuji Film, Tokyo, Japan) and ImageJ software (NIH, Bethesda, MD, USA).

Transfection of siRNA. To confirm the effects of GRP78 knockdown on apoptosis, we examined GRP78 knockdown by small-interfering RNA (siRNA). Cells were transfected with 50 nM si-GRP78 or si-control, using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol (20). After 24 h of transfection, cells were treated with 1 µM thapsigargin for 24 h.

Statistical analysis. Results are expressed as means ± standard deviation (SD). Statistical analysis was performed using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Human pancreatic cancer cell lines express GRP78. First, we examined the GRP78 expression in the human pancreatic cancer cell lines SUIT-2, MIAPaCa-2, Panc-1 and KP-2 (3). Protein samples were collected from the four pancreatic cancer cell lines, and protein levels of GRP78 were investigated by western blotting with a specific antibody for GRP78 (Fig. 1). We confirmed that all four pancreatic cancer cell lines variably expressed GRP78.

Thapsigargin upregulates the protein levels of GRP78 in the human pancreatic cancer cell lines. Next, we examined the effect of thapsigargin, one of the ER stress-inducers, on GRP78 expression in the human pancreatic cancer cell lines (Fig. 2). Treatment of 1 µM thapsigargin for 24 h led to the upregulation of GRP78 expression at the protein level [21.5±0.7 vs. 1±0.1 (in untreated control), n=3, p=0.00015; 11.1±1.0 vs. 1±0.12, n=3, p=0.000010; 5.2±0.57 vs. 1±0.1, n=3, p=0.0023; and 5.9±0.2 vs. 1±0.1, n=3, p=0.00013, respectively, in the SUIT-2, MIAPaCa-2, Panc-1 and KP-2 cells]. In the MIAPaCa-2, cells GRP78 expression was more strongly induced than in the other three cell lines.
Effects of thapsigargin on GADD34, ATF4, ATF6 and XBP1 protein expression levels in the human pancreatic cancer cell lines.

We examined the protein expression of ER stress signaling-associated molecules in the human pancreatic cell lines treated with or without thapsigargin. The results for the Panc-1 and KP-2 cells are shown in Fig. 3. In the Panc-1 cells, ATF4 and ATF6 expression was upregulated in the presence of 1 µM thapsigargin [1.4±0.010 vs. 1±0.023 (in untreated control), n=3, p=0.000089; and 1.2±0.0027 vs. 1±0.010, n=3, p=0.00019, respectively] (Fig. 3A, C and D. In the Panc-1 cells, GADD34 and XBP1 expression at the protein level was downregulated in the presence of 1 µM thapsigargin [0.82±0.012 vs. 1±0.0076 (in untreated control), n=3, p=0.0000414; and 0.87±0.024 vs. 1±0.019, n=3, p=0.0012, respectively] (Fig. 3A, B and E).

On the other hand, in KP-2 cells, the protein expression levels of GADD34, ATF4, ATF6 and XBP1 were upregulated in the presence of 1 µM thapsigargin [2.1±0.22 vs. 1±0.012 (in untreated control), n=3, p=0.00063; 1.4±0.073 vs. 1±0.0062, n=3, p=0.00088; 2.1±0.022 vs. 1±0.014, n=3, p=0.0000008; and 1.2±0.019 vs. 1±0.0063, n=3, p=0.00043, respectively] (Fig. 3A, B and E).

XBP1 was also upregulated in the presence of 1 µM thapsigargin in both SUIT-2 and MIAPaCa-2 cells, yet we did not observe any enhancement of GADD34, ATF4 or ATF6 by thapsigargin (data not shown).

Effects of thapsigargin on the phosphorylation of eIF2α in the human pancreatic cancer cell lines. We also examined the protein expression of ER stress signaling-associated molecules in the human pancreatic cell lines treated with or without thapsigargin. The results for the Panc-1 and KP-2 cells are shown in Fig. 3. In the Panc-1 cells, ATF4 and ATF6 expression was upregulated in the presence of 1 µM thapsigargin [1.4±0.010 vs. 1±0.023 (in untreated control), n=3, p=0.000089; and 1.2±0.0027 vs. 1±0.010, n=3, p=0.00019, respectively] (Fig. 3A, C and D. In the Panc-1 cells, GADD34 and XBP1 expression at the protein level was downregulated in the presence of 1 µM thapsigargin [0.82±0.012 vs. 1±0.0076 (in untreated control), n=3, p=0.0000414; and 0.87±0.024 vs. 1±0.019, n=3, p=0.00012, respectively] (Fig. 3A, B and E).

On the other hand, in KP-2 cells, the protein expression levels of GADD34, ATF4, ATF6 and XBP1 were upregulated in the presence of 1 µM thapsigargin [2.1±0.22 vs. 1±0.012 (in untreated control), n=3, p=0.00063; 1.4±0.073 vs. 1±0.0062, n=3, p=0.00088; 2.1±0.022 vs. 1±0.014, n=3, p=0.0000008; and 1.2±0.019 vs. 1±0.0063, n=3, p=0.00043, respectively] (Fig. 3A, B and E).

XBP1 was also upregulated in the presence of 1 µM thapsigargin in both SUIT-2 and MIAPaCa-2 cells, yet we did not observe any enhancement of GADD34, ATF4 or ATF6 by thapsigargin (data not shown).
In the KP-2 cells, significant phosphorylation of Ser51-eIF2α in the presence of thapsigargin was observed when compared with that in the absence of thapsigargin (Fig. 4C; 2.1±0.14 vs. 1±0.075, n=3, p=0.00050).

Knockdown of endogenous GRP78 enhances PARP cleavage in the pancreatic cancer cells. We confirmed that the expression of GRP78 at the protein level was upregulated in all four human pancreatic cancer cell lines tested, yet other molecules downstream of GRP78 reported to be involved in ER stress were expressed at variable levels depending on the individual cell line. Thus, we focused our examination on GRP78. Our previous study (13) demonstrated that blocking of GRP78 induction led to PARP cleavage in hepatocyte apoptosis. We investigated the effect of knockdown of GRP78 by siRNA on PARP cleavage in pancreatic cancer cells treated with thapsigargin (Fig. 5A and B).

GRP78 expression was significantly inhibited by transfection with si-GRP78 in the presence of thapsigargin, compared with that with si-control [1.4±0.040 vs. 1.8±0.040, n=3, p=0.00014; and 7.1±0.24 vs. 18.3±0.37, n=3, p=0.0000038, respectively, in Panc-1 (Fig. 5A) and MIAPaCa-2 cells (Fig. 5B)].

PARP cleavage was significantly enhanced by transfection with si-GRP78 in the presence of thapsigargin, compared with that with si-control [4.5±0.045 vs. 1.6±0.085, n=3, p=0.0000080; and 2.6±0.13 vs. 1.5±0.047, n=3, p=0.00016, respectively, in Panc-1 (Fig. 5A) and MIAPaCa-2 cells (Fig. 5B)].

**Discussion**

In the present study, we demonstrated that i) human pancreatic cancer cell lines expressed GRP78; ii) ER stress induced by thapsigargin upregulated protein levels of GRP78 in human pancreatic cancer cell lines; iii) ER stress-related molecules downstream of GRP78 were expressed at various levels according to the respective human pancreatic cancer
cell lines; and iv) finally, knockdown of GRP78 by siRNA enhanced PARP cleavage in the human pancreatic cancer cell lines. To our knowledge, this is the first report to show the association between GRP78 and PARP cleavage in pancreatic cancer cell lines treated with thapsigargin.

Our results that human pancreatic cancer cell lines express GRP78 supported a previous study (21) showing that the heat shock proteins HSP90 and GRP78 are constitutively expressed in gastrointestinal cancers including human pancreatic cancer. We also observed that ER stress induced by thapsigargin upregulated protein levels of GRP78 in human pancreatic cancer cell lines. However, ER stress-related molecules downstream of GRP78, such as GADD34, ATF4, ATF6, XBP1 and phospho-eIF2α were not constitutively increased by thapsigargin, but rather were dependent on individual cell lines (Figs. 2-4). These results suggest that GRP78 may have an impact on many different cellular processes and survival of pancreatic cancer and that ER stress signaling downstream of GRP78 can be expected to be disturbed in pancreatic cancer.

It was reported that an increase in GRP78 expression in pancreatic cancer cells may enhance and account for the altered sensitivity of pancreatic cancer to chemotherapeutic agents (21). UPR regulator GRP78 is an anti-apoptotic protein that is usually upregulated in cancer and plays a critical role in chemo-resistance in various types of cancers (22). Recently it was also reported that UPR induction in tumor endothelial cells under an acidic pH condition is related to chemoresistance and may contribute to therapeutic failure in response to chemotherapy (23). It was also reported that GRP78 is overexpressed in malignant cells resistant to therapy (24).

PARP is one of the proteins processed by post-translational modification and plays a crucial role in many processes, including DNA repair and cell death (25). During apoptosis, caspases cause PARP cleavage and inactivation, in which PARP proteolysis produces an 89-kDa C-terminal fragment and a 24-kDa N-terminal (25). We observed that in the presence of thapsigargin, knockdown of GRP78 enhanced PARP cleavage in human pancreatic cancer cells Panc-1 as well as MIAPaCa-2. Wang et al reported that suppression of GRP78 by taxol and vinblastine potentiated the activation of JNK phosphorylation, caspase-7 and PARP cleavage in the human breast cancer cell line MCF-7 (26). The Hsp90 inhibitor SNX-2112 also induced PARP cleavage as well as the reduction in GRP78 expression in the multidrug-resistant human chronic myeloid leukemia K562/ADR cell line (27).

Collectively, our results suggest that both GRP78 and PARP may have key roles in the chemoresistance of pancreatic cancer (28) and that GRP78 may be one of the valid targets against chemoresistance (24). In conclusion, GRP78 is a potential therapeutic target for ‘difficult-to-treat’ pancreatic cancer, in which ER stress signaling in part falls into disorder.

Acknowledgements

The present study was supported by Grants for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (24590955 to T.K.).

References

1. Lennon AM, Wolfgang CL, Canto MI, et al: The early detection of pancreatic cancer: will it take to diagnose and treat curable pancreatic neoplasia? Cancer Res 74: 3381-3389, 2014.
2. Kanda T, Kumagi T, Takada T, et al: Improvement of long-term outcomes in pancreatic cancer and its associated factors within the gemcitabine era: a collaborative retrospective multicenter clinical review of 1,082 patients. BMC Gastroenterol 13: 134, 2013.
3. Okitsu K, Kanda T, Imazeki F, Yonemitsu Y, Ray RB, Chang C and Yokosuka O: Involvement of interleukin-6 and androgen receptor signaling in pancreatic cancer. Genes Cancer 1: 859-867, 2010.
4. Miyazaki M, Yoshitomi H, Shimizu H, et al: Repeat pancreatectomy for pancreatic ductal cancer recurrence in the remnant pancreas after initial pancreatectomy: is it worthwhile? Surgery 155: 58-66, 2014.
5. Sudo K, Ishihara T, Hirata N, et al: Randomized controlled study of gemcitabine plus S-1 combination chemotherapy versus gemcitabine for unresectable pancreatic cancer. Cancer Chemother Pharmacol 73: 389-396, 2014.
6. Mahadevan NR, Rodvold J, Sepulveda H, Rossi S, Drew AF and Zenetti M: Transmission of endoplasmic reticulum stress versus gemcitabine for unresectable pancreatic cancer. Cancer Chemother Pharmacol 73: 389-396, 2014.
7. Schroder M and Kaufman R: ER stress and the unfolded protein response. Nat Rev Cell Biol 6: 1129-1134, 2005.
8. Hamaanaka RB, Bennett BS, Cullinan SB and Dihl JA: PERK and GCN2 contribute to eIF2α phosphorylation and cell cycle arrest after activation of the unfolded protein response pathway. Mol Cell 16: 5493-5501, 2005.
9. Xu W, Liu L, Charles IG and Moncada S: Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response. Nat Cell Biol 6: 1129-1134, 2004.
10. Ma Y and Hendershot LM: The role of the unfolded protein response in tumor development: friend or foe? Nat Rev Cancer 4: 966-977, 2004.
11. Jiang X, Kanda T, Nakamoto S, Miyamura T, Wu S and Yokosuka O: Involvement of androgen receptor and glucose-regulated protein 78 kDa in human hepatocarcinogenesis. Exp Cell Res 323: 326-336, 2014.
12. Shuda M, Kondoh N, Imazeki N, et al: Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J Hepatol 38: 605-614, 2003.
13. Jiang X, Kanda T, Tanaka T, Wu S, Nakamoto S, Imazeki F and Yokosuka O: Lipopolysaccharide blocks induction of unfolded protein response in human hepatoma cell lines. Immunol Lett 152: 8-15, 2013.
14. Martinon F and Glimcher LH: Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum. Curr Opin Immunol 23: 35-40, 2011.
15. Kanda T, Jiang X and Yokosuka O: Androgen receptor signaling in hepatocellular carcinoma and pancreatic cancers. World J Gastroenterol 20: 9229-9236, 2014.
16. Nawrocki ST, Carew JS, Dunner K Jr, et al: Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. Cancer Res 65: 11510-11519, 2005.
17. Mujumdar N, Banerjee S, Chen Z, et al: Triptolide activates unfolded protein response leading to chronic ER stress in pancreatic cancer cells. Am J Physiol Gastrointest Liver Physiol 306: G1011-G1020, 2014.
18. Fu Y and Lee AS: Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. Cancer Biol Ther 5: 741-744, 2006.
19. Lee E, Nichols P, Spencer D, Grosven R, Mc Y and Lee AS: GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer Res 66: 7849-7853, 2006.
20. Kanda T, Yokosuka O, Imazeki F, Arai M and Saisho H: Enhanced sensitivity of human hepatoma cells to 5-fluorouracil by small interfering RNA targeting Bcl-2. DNA Cell Biol 24: 805-809, 2005.
21. Ehrenfried JA, Herron BE, Townsend CM Jr and Evers BM: Heat shock proteins are differentially expressed in human gastrointestinal cancers. Surg Oncol 1: 197-203, 1995.
22. Tsai HY, Yang YF, Wu AT, et al: Endoplasmic reticulum ribosome-binding protein 1 (RRBP1) overexpression is frequently found in lung cancer patients and alleviates intracellular stress-induced apoptosis through the enhancement of GRP78. Oncogene 32: 4921-4931, 2013.
23. Visioli F, Wang Y, Alam GN, Ning Y, Rados PV, Nör JE and Polverini PJ: Glucose-regulated protein 78 (Grp78) confers chemoresistance to tumor endothelial cells under acidic stress. PLoS One 9: e101053, 2014.
24. Roller C and Maddalo D: The molecular chaperone GRP78/BiP in the development of chemoresistance: mechanism and possible treatment. Front Pharmacol 4: 10, 2013.
25. Soldani C and Scovassi AI: Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis 7: 321-328, 2002.
26. Wang J, Yin Y, Hua H, et al: Blockade of GRP78 sensitizes breast cancer cells to microtubules-interfering agents that induce the unfolded protein response. J Cell Mol Med 13: 3888-3897, 2009.
27. Wang R, Shao F, Liu Z, et al: The Hsp90 inhibitor SNX-2112, induces apoptosis in multidrug resistant K562/ADR cells through suppression of Akt/NF-κB and disruption of mitochondria-dependent pathways. Chem Biol Interact 205: 1-10, 2013.
28. Lei Y, Henderson BR, Emmanuel C, Harnett PR and Defazio A: Inhibition of ANKRD1 sensitizes human ovarian cancer cells to endoplasmic reticulum stress-induced apoptosis. Oncogene: Feb 17, 2014 (Epub ahead of print).