Selective Cytotoxicity of Ponciri Fructus against Glucose-Deprived PANC-1 Human Pancreatic Cancer Cells via Blocking Activation of GRP78

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Received April 2, 2009; Accepted July 27, 2009; Online Publication, October 7, 2009

Pancreatic cancer cells are sometimes exposed to stressful microenvironments such as glucose deprivation, hypoxia, and starvation of other nutrients. These stresses, which are characteristic of poorly vascularized solid tumors, activate the unfolded protein response (UPR). The UPR is a stress-signaling pathway present in tumor cells that is associated with molecular chaperone GRP78. Induction of GRP78 has been found to increase cell survival and decrease apoptotic potential through genetic alterations. Thus GRP78 may represent a novel target in the development of anticancer drugs. Here we established a novel screening program to identify chaperone modulators that exhibit preferential cytotoxic activity in glucose-deprived pancreatic cancer cells. During the course of our screening, we isolated an active substance, Ponciri Fructus (PF), from an herbal medicine source and identified it as a down-regulator of GRP78. As expected, PF inhibited expression of the GRP78 protein under glucose-deprivation conditions in a dose-dependent manner. Furthermore, it induced selective cytotoxicity against glucose-deprived cancer cells; this effect was not observed under normal growth conditions. We also detected apoptotic bodies on Hoechst staining and attempted to determine whether PF-induced apoptosis involved caspase-3 activation. Our results suggest that the GRP78-inhibitory action of PF was dependent on strict hypoglycemic conditions and that it resulted in the selective death of glucose-deprived pancreatic cancer cells.

Key words: pancreatic cancer; glucose deprivation; unfolded protein response; GRP78; Ponciri Fructus

Pancreatic cancer is a disease in which malignant cancer cells form in tissues of the pancreas. About 90% of all pancreatic cancers are ductal adenocarcinomas with an overall 5-year survival rate of less than 5%.1,2) The reason for this high mortality rate is explained by the fact that pancreatic cancer is difficult to detect and diagnose, since there are no noticeable signs or symptoms in the early stages of the disease. Furthermore, the pancreas lies “hidden” behind other organs, such as the stomach, small intestine, and bile duct. For these reasons, pancreatic cancer is one of the most lethal human malignancies, and is the forth leading cause of cancer-related death in adults.3) Treatment of pancreatic cancer is an important medical problem. At present, it is treated by combining surgery with radiation therapy and chemotherapy, although surgery remains the principal treatment, but, these therapeutic approaches have severe shortcomings.4,5) Therefore, novel anticancer agents possessing specific pancreatic cell activity are needed in order to treat this cancer more effectively. Based on these facts, we established a new molecular target in the development of selective chemotherapeutic drugs that focus on the peculiarities of pancreatic cancer.

The typical solid tumors of pancreatic cancer are often exposed to stress conditions, such as glucose deprivation.6,7) These microenvironmental conditions are based primarily on inadequate vascularization, and they favor tumor cell survival, and are not present in normal cells.8–10) These physiological stresses also induce activation of the unfolded protein response (UPR) pathway. The UPR is a conserved, adaptive cellular program that is activated in response to an accumulation of unfolded proteins in the endoplasmic reticulum (ER).11–13) Several genes are up-regulated upon UPR activation, such as glucose-regulated protein (GRP) 78 (also known as BiP) and ER-resident molecular chaperones.14,15) Recently, several reports have indicated that GRP78 plays a role in protecting tumor cells against intracellular-mediated cytotoxicity.

In a variety of solid tumors and human cancer cell lines, GRP78 levels are elevated and appear to correlate with malignancy. In addition, GRP78 induction has been found to protect cancer cells from immune surveillance, whereas suppression of stress-mediated GRP78 induction enhances apoptosis-inhibited tumor growth and increases cytotoxicity.16–18) Hence down-regulation of GRP78 in pancreatic cancer cells under glucose-deprivation conditions is of potential use in cancer therapy.

Hence recently we shows screened 435 herbal medicine varieties, and determined that Ponciri Fructus (PF) exerts selective cytotoxicity against human pancreatic cancer cells under glucose deprivation. Here we investigated the effect of PF on GRP78 activation in cells exposed to glucose-deprivation conditions. We also examined the role of apoptosis in the selective cytotoxicity of PF in glucose-deprived PANC-1 human pancreatic cancer cells.

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Abbreviations: UPR, unfolded protein response; PF, Ponciri Fructus; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; LDH, lactate dehydrogenase
Materials and Methods

Materials and preparation of methanolic extracts from PF. Ponciri Fructus was kindly supplied by Kumkang Pharm. (Masan, South Korea). Five g of PF was extracted with 100 ml of methanol over 3 d at room temperature and filtered through Whatman No. 1 filter paper (Advantec, Tokyo). The methanolic extract from PF was denoted PFM. The PFM was then dissolved in dimethyl sulfoxide (DMSO) and stored at −20° C. Then it was added to the cell culture medium so that the DMSO made up less than 0.5% of the total volume of the culture.

Cell culture and treatments. PANC-1 human pancreatic adenocarcinoma cells were obtained from the Korean Cell Line Bank (KCLB). The cells were maintained in DMEM medium containing 2 mg of glucose/ml (Invitrogen, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 3.7 mg/ml of NaHCO3 in a humidified incubator set at 37° C and 5% CO2. Glucose deprivation was achieved by growing the cells in glucose-free DMEM medium (Invitrogen) supplemented with 10% heat-inactivated FBS, as described previously.50

Cell viability assay. Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). An LDH release assay kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). PANc-1 cells were seeded in 96-well plates (1.5 × 103 cells per well), cultured overnight in the presence and the absence of glucose, and treated with various concentrations of PFM for 24 h, as described above. The LDH assay reaction was initiated in a 96-well plate by mixing 50 μl of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 μl. A colorimetric assay was performed, in which the amount of formazan salt was proportional to the level of LDH activity in the sample. The intensity of the resulting red color measured at 540 nm was therefore proportional to LDH activity.

Colony formation assay. PANc-1 cells were seeded at 5.0 × 103 cells/ml in 24-well plates, and incubated overnight, followed by culture in medium with and without glucose for 24 h. The cells were subsequently diluted in new medium, replated at 1.0 × 103 cells/ml in 6-well plates, and cultured to form colonies under normal growth conditions for 7 or 8 d at 37° C in a humidified atmosphere containing 5% CO2. Colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted.50

Immunoblot analysis. PANc-1 cells were lysed in 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tri-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol), as previously described.50 Equal amounts of proteins were resolved on a 5–20% SDS-polyacrylamide gel and transferred by electrophoresis onto a nitrocellulose membrane. Immunoblots were probed with the following primary antibodies: mouse monoclonal anti-KDEL (for detection of GRP78; StressGen, Victoria, Canada); rabbit polyclonal anti-CREB-2 (ATF4; Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin (an internal control; Sigma); and rabbit polyclonal anti-caspase-3 (Cell Signaling, Danvers, MA). Membranes were incubated for 2 h with the above primary antibodies under similar conditions. The membranes were washed with PBS containing 5% nonfat dry milk at room temperature and incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulin (Amersham Pharmacia Biotech, Tokyo) under similar conditions. After washing with PBS, specific signals were detected with an enhanced chemiluminescence detection system (Amersham).

Measurement of apoptosis and cell staining. Flow cytometric analysis of cellular DNA content was performed as described previously (Park et al., 2004). Briefly, PANc-1 cells were harvested and fixed with ice-cold 70% ethanol. The fixed cells were stained with 50 μg/ml of propidium iodide at room temperature in the dark for 30 min. Apoptotic cells were counted using a FACs caliber flow cytometer (Becton Dickinson, San Jose, CA). The morphology of the apoptotic cells was investigated by staining them with Hoechst 33342 (Sigma). PANc-1 cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 10% formaldehyde for 2 h at room temperature the fixed cells were washed with PBS and stained with Hoechst 33342 for 1 h at room temperature. They were washed twice more with PBS, and the Hoechst-stained nuclei were visualized with a fluorescence microscope.

Statistical analysis. All data presented are the means of three determinations. Data were analyzed using the SPSS package for Windows (Version 11.5; Chicago, IL) and evaluated by one-way analysis of variance (ANOVA) followed by Scheffe’s test. Differences were considered significant at p < 0.05.

Results

Survival response to glucose deprivation in pancreatic cancer cells

To determine the degree of tolerance of pancreatic cancer cells to glucose deprivation, we investigated the extent of PANc-1 cell survival under extremely low glucose conditions. As shown in Fig. 1A, PANc-1 cells survived for extensive periods in the presence and the absence of glucose. In addition, cell viability was determined by colony formation and LDH release assays. The results showed resistance to glucose shortage in the present state, as expected (Fig. 1B and C). Pancreatic cancers have the ability to grow under glucose depriva-
tion, as shown in Fig. 1A, B, and C and as confirmed by biological experimentation. These observations are in agreement with the hypothesis that pancreatic cancer cells in vivo survive in stressful microenvironments, as well as in hypoxic conditions at all times.

This result was revealed in accordance with UPR. Considering this, we determine whether pancreatic cancer cells would be able to endure low-glucose conditions. To establish a link among cell survival, glucose-deprivation conditions, and activation of the UPR, we performed western blot analysis on PANC-1 cells that had been subjected to glucose-deprivation conditions. Under the experimental conditions described above, glucose deprivation induced expression of GRP78 in PANC-1 cells in a time-dependent manner (Fig. 1D). We also found that glucose deprivation led to up-regulation of the expression of ATF4, a downstream transcription factor in the UPR signaling pathway (Fig. 1D). These results indicate that the expression of certain UPR target proteins is correlated with survival in cancer cells under glucose-deprivation conditions and that PANC-1 cells possess a strong survival capacity, even under conditions of glucose shortage.

**PFM-induced selective cytotoxicity against glucose-deprived PANC-1 cells**

In the course of a recent screening of 435 herbal medicine extracts, we isolated an active substance from Ponciri Fructus (PF) that preferentially exerted cytotoxic activity in glucose-deprived PANC-1 cells. The effects of methanolic extracts of PF on PANC-1 morphological alterations were determined by phase-contrast microscopy. As shown in Fig. 2A, after 48 h of incubation with 10 and 50 μg/ml of PFM in the presence and the absence of glucose, the methanolic extract exhibited no cytotoxic effects on cell morphology under normal growth conditions, but, when PFM was administered to cells cultured in a glucose-deprived medium, many cells exhibited cytoplasmic shrinkage and either detached from each other or floated in the medium.

Next, to assess the selective cytotoxicity induced by PFM in PANC-1 cells under low-glucose stress conditions, cells were incubated with PFM as described above and cytotoxicity was determined by LDH and colony formation assays (Fig. 2B and C). As shown in Fig. 2B, under normal growth conditions, a 24 h treatment with PFM had little effect on PANC-1 cell viability. In contrast, cells exposed to PFM at 50 μg/ml showed 2-fold increase in cytotoxicity as compared with the glucose-free stress controls. A colony formation assay further revealed that PFM treatment was highly toxic to PANC-1 cells in the glucose-free medium, with a half maximal inhibitory concentration (IC50) of 10 μg/ml (Fig. 2C). These results suggest that PFM is not cytotoxic to PANC-1 cells under normal growth conditions, but that it preferentially reduces pancreatic cancer cell viability under glucose-deprivation conditions.

**GRP78-inhibiting and apoptosis inducing activities of PFM under glucose deprivation**

To examine a possible correlation between PFM cell-mediated selective cytotoxicity and GRP78-inhibiting activity, we performed a western blot analysis of PANC-1 cells that had been subjected to glucose deprivation

![Fig. 2. Selective Cytotoxicity of Methanolic Extracts of Ponciri Fructus (PFM) in Glucose-Deprived PANC-1 Cells.](image)

A, Morphological changes in glucose-deprived PANC-1 cells: a, normal conditions; b, normal conditions/10 μg/ml PFM; c, normal conditions/50 μg/ml PFM; d, glucose deprivation; e, glucose deprivation/10 μg/ml PFM; f, glucose deprivation/50 μg/ml PFM. The photographs were taken with a phase-contrast microscope at 100× magnification. B, Cell viability was measured by LDH release assay in cells exposed to PFM under normal and glucose-deprivation conditions. C, Colony formation analysis of PANC-1 cells exposed to the indicated concentrations of PFM for 24 h in the presence and the absence of glucose. After colony formation, the survival rate was calculated by setting each of the control survival rates. Glc: glucose. *p < 0.05; **p < 0.01; ***p < 0.001 compared with normal growth control. #p < 0.01; ##p < 0.001 as compared with glucose deprivation control.

![Fig. 3. PFM Down-Regulated GRP78 Expression and Induced Caspase-3 Activation in Glucose-Deprived PANC-1 Cells.](image)

Cells were treated with PFM at the indicated concentrations for 24 h. Total cell lysates of PANC-1 cells were prepared and subjected to western blot analysis. Protein samples (10 μg/lane) were separated by SDS–PAGE, and expression levels of GRP78 and caspase-3 were assessed in PANC-1 cells. β-actin was measured as an internal loading control. **p < 0.01; ***p < 0.01 compared with normal growth control. #p < 0.01 compared with glucose deprivation control.
for 24 h, in the presence and the absence of PFM. As expected, PFM suppressed the accumulation of GRP78 under glucose deprivation but had no effect on the expression level of GRP78 under normal growth conditions (Fig. 3). To determine the potential involvement of the survival signal pathway in the PFM mechanism of cell death, we verified the expression level of caspase-3 (a protein that plays a central role in apoptosis) in our system. Treatment of glucose-deprived cells with 10 and 50 μg/ml of PFM for 24 h induced dose-dependent cleavage of caspase-3 to its 19 kDa active form (Fig. 3). In this way, down-regulation of GRP78 expression, which operates as a key factor in the UPR survival signal pathway, by PFM led to inhibition of tumor growth in glucose-deprived PANC-1 cells by activating selective apoptosis mechanisms associated with caspase-3 cleavage.

To investigate the mechanism of cell death induced by PFM under glucose-deprivation conditions, we examined the apoptotic activity of PANC-1 cells in response to PFM treatment glucose-free conditions using Hoechst 33342 staining and FACS analysis. We collected cells after PFM treatment in the presence and the absence of glucose and examined nuclear morphological changes. PANC-1 cells treated with 10 and 50 μg/ml of PFM for 48 h showed marked chromatin condensation and formation of apoptotic bodies under glucose-deprivation conditions but not under normal growth conditions, indicating selective induction of apoptosis in PFM-treated glucose-deprived cancer cells (Fig. 4A). We also tried to determine cells would be similarly sensitized to glucose-deprivation conditions by PFM treatment using flow cytometry assay of apoptotic cells distributed according to cell cycle stage and showing sub-G1 DNA content. The number of apoptotic cells in the sub-G1 phase increased approximately 15-fold in the presence of 50 μg/ml of PFM as compared with the control cells (Fig. 4B). This result also indicated a depletion of cells in the G1 phase and a concomitant accumulation of cells in the G2/M phase, induced by glucose-deprivation conditions. This arrest in the G2/M phase was also accompanied by an increase in the number of sub-G1 cells, which is typical of the late stages of apoptosis. These results suggest that glucose-deprived PANC-1 cells are susceptible to a dramatically selective cytotoxicity effect, and that these antiproliferative outcomes are not present under normal glucose conditions.

**Discussion**

Solid tumors, especially pancreatic cancer, are refractory to chemotherapy due to their incomplete vascularization, and they are often surrounded by stressful microenvironments, including glucose deprivation, hypoxia, low pH, and other nutrient deprivations, which are not commonly observed in normal tissues. In addition, these microenvironmental stress conditions can lead to selection of tumor cells that have decreased apoptotic potential through genetic alterations, leading
to resistance to apoptosis. Physiological stresses in tumor cells, such as glucose deprivation, can disrupt protein folding in the ER and lead to activation of the molecular chaperone GRP78, which is associated with the UPR stress signaling pathway. GRP78 plays a role in protecting tumor cells against intracellular-mediated cytotoxicity and against the toxic effects of anticancer agents in vitro, suggesting that GRP78 induction also protect tumor cells in vivo. The levels of GRP78 are elevated in solid tumors, and induction of GRP78 has been found to protect cancer cells from immune surveillance, whereas suppression of stress-mediated induction of GRP78 enhances apoptosis, inhibits tumor growth, and increases cytotoxicity in chronically glucose-deprived cells. As we achieve a greater understanding of the mechanisms and physiological roles of GRP78, it should be possible to design novel chemotherapies for cancer targeting this molecule.

In our earlier studies, we discovered a novel compound, versipelostatin (VST), that exhibits selective cytotoxic activity in vitro and an antitumor effect in vivo against human colon cancer cells during glucose deprivation. One clear biochemical effect of VST is that it can specifically suppress GRP78 expression in response to glucose deprivation in cancer cells. In our current study, we found that down-regulation of GRP78 induced selective cytotoxicity in glucose-deprived PANC-1 pancreatic cancer cells. Using microscopy and viability assays, we found that pancreatic cancer cells maintained growth even extremely low-glucose conditions. As expected, Western blotting analysis also confirmed that survival proteins of the UPR complex, such as GRP78 and ATF-4, were elevated in glucose-deprived PANC-1 cells. Thus, tumor growth and progression appear to depend largely on glucose-deprivation conditions, as well as other physiological stresses. Targeting cancer cells under glucose-deprivation conditions can therefore be considered an attractive approach to cancer chemotherapy. To confirm the presence of selective cytotoxicity in glucose-deprived pancreatic cancer cells, we identified the PF compound from an oriental medicine. PF exhibits highly selective cytotoxicity to glucose-deprived PANC-1 cells. This compound was also found to be associated with inhibition of GRP78 expression in glucose-deprived PANC-1 cells. Moreover, down-regulation of GRP78 can induce apoptosis involving a mitochondrial pathway by activation of caspase-3. Finally, PF treatment led to cell cycle arrest as evidenced by an increase in the number of cells with fractional DNA content in the sub-G1 region.

PF exhibited no cytotoxic effect under normal growth conditions, when there was an adequate glucose supply in the culture medium. The clear cytotoxic effect of PF in PANC-1 cells was evident only during glucose deprivation. This study indicates that PF is a candidate for a novel therapeutic approach that induces selective pancreatic cancer cell death via GRP78 down-regulation. In conclusion, selective inhibitors of glucose-deprived pancreatic cancer cell growth, such as PF, are good candidates to explore potential cancer targets and therapies. We believe that the current study provide a promising new therapeutic strategy for the treatment of pancreatic cancer.

Acknowledgments

This work was supported by Kyungnam University Foundation Grant, 2007.

References